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AN EXPERIMENTAL STUDY ON GROWTH AND GLYCOLYSIS IN THE EPIPHYSEAL CARTILAGE OF RATS

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UNIVERSITY OF MICHIGAN

ACTA PHYSIOLOGICA SCANDINAVICA
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FROM THE JOHAN THRONE HOLST'S
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NICOLAY EEG-LARSEN

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Foreword.

The experimental work described in the present publication has been carried out at Johan Throne Holst's Nutritional Research Institute, the University of Oslo.

I am greatly indepted to the head of the Institute, Professor Ragnar Nicolaysen who has provided me with excellent working facilities. His interest and encouragement has been a constant source of inspiration.

I also wish to thank Miss Butte Wikandt, Mrs. Anne Lise Kristoffersen and Mrs. Jean Jung for valuable technical assistance.

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Oslo, February 1956.

Nicolay Eeg-Larsen

Introduction.

Most mammalian bones develop from preformed cartilage. The endochondral bone formation is accompanied by characteristic morphological changes in the structure of the cartilage in those areas where cartilage is replaced by the newly formed bone tissue. The most typical changes take place in the cartilaginous epiphyseal plate which in growing bones separates the diaphysis from the epiphysis. The primary function of the epiphyseal plate is to support the longitudinal growth of young bones.

Bone salts are first precipitated in those areas of the cartilaginous plate bordering the diaphyseal bone tissue. This primary calcification of the cartilage has been subjected to a great number of investigations so as to gain knowledge about the processes responsible for the local precipitation of the inorganic components. They may be grouped as follows:

1. Studies of the composition and structure of bone salts.
2. Studies of the composition of serum and of tissue fluids with respect to the bone minerals under pathological and experimental conditions resulting in changes in the mineralization of bone.
3. Physico-chemical studies of the solubility and saturation products in serum and in synthetic model systems.
4. Studies of the ion-binding capacities of the organic ground substance of bone.
5. Studies of the enzymatic and metabolic processes in cells and tissues in the calcifying areas.

Each of these studies has provided valuable information about the composition and structure of the bone salts, the properties of the tissue as well as the cellular and enzymatic processes in the calcifying areas.

No definite and satisfactory explanation of the mechanism underlying the precipitation and the deposition of bone salts, however, has been offered. The results from a number of investigations employing different procedures are suggestive of an intimate connection between the distribution of glycogen and the primary deposition of bone salts in the calcifying areas of the cartilage. The existence of such a connection is only indirectly indicated. The carbohydrate metabolism in the epiphyseal plate has been subjected to only a few studies. In the course of the last 20 years knowledge of intermediary carbohydrate metabolism has been greatly enlarged. At the present day every single stage in the gradual decomposition of glycogen and glucose to lactic acid is known and the majority of the enzymes that catalyze the separate steps have been isolated.

The primary object of the present work has been to study the intermediary carbohydrate metabolism in the epiphyseal cartilage *in vitro*. A quantitative expression for the activity of the glycolytic process in epiphyseal cartilage was the original aim. It followed naturally from these measurements to investigate the activity of the separate stages in glycogenolysis and hence ascertain, if possible, whether any single stage was rate limiting.

In the course of the work, it was found necessary to supplement the originally planned investigations with others on the rate of growth of the bone and the content of glycogen and lactic acid in bones at different ages.

The tibia from rat was chosen for practical reasons. In the first place rats are readily obtainable in adequate numbers. Secondly the rate of growth of the rat tibia and the histological structure of the proximal epiphyseal plate have been well described. Thirdly this bone is easily dissected free from soft tissue and it is a comparatively simple matter to cut the relatively plane epiphyseal plate free from adjacent bone-forming elements.

Evaluation of results from metabolic and histochemical investigations of bone growth and calcification should be based on the characteristic histological structure of the epiphyseal plate. It has therefore been judged necessary to recapitulate the most important morphological features of the endochondral bone formation in an introductory chapter.

PART I. GENERAL REVIEW

Chapter I.

The Histology of the Endochondral Bone Formation.

The anlage of the future bone is a crude, small scale model consisting of cartilage. The cartilage cells are not all of equal size. They hypertrophy in the central areas and form in columns towards the ends. In the zones nearer the ends the cells are flat and the many visible mitoses indicate active cell division. The remaining cartilage contains the usual small cells, evenly distributed in the hyaline ground substance. At a later stage of development the initial calcified tissue appears in a thin collar in the space between the perichondrium and the hypertrophic cells corresponding to the central section. This perichondral collar is surrounded by the periosteum, hitherto known as the perichondrium. In the succeeding development, buds of vascular tissue penetrate from openings in the collar into the hypertrophic cartilage cells at several points simultaneously and in front of them large polycyclic lacunae are hollowed out of the cartilage. This stage is characterized by the appearance of processes leading to the destruction of the hypertrophic cartilage cells. Primary bone marrow develops in the invading tissue and the newly formed bone trabeculae are continuous with the cartilaginous epiphyses. A small bone with all its typical components has now been formed. The central part consists of a small osseous tube, the diaphysis, filled with embryonic bone marrow between the primary bone trabeculae. Both ends, the epiphyses, still consists entirely of hyaline cartilage. When the formation of a bony shaft is well advanced, one or more centres of ossification appear in each ex-

tremity of the long bone. The cells in the central areas of the epiphyses hypertrophy, vascularized tissue invades from the perichondrium, and similarly to the development in the diaphysis, the cartilage is gradually resorbed and replaced by spongy bone. The latter which has now been formed in both the diaphysis and the epiphysis, is separated by a plate of cartilage, called the epiphyseal plate.

The epiphyseal plate has a characteristic histological structure and can be divided into three zones according to the arrangement and size of the cells.

1. *Cell Division Zone.* Close to the bony epiphysis is a narrow zone consisting of irregularly arranged embryonic cartilage cells. These are small with little cytoplasm and are scattered in an ample hyaline matrix. Mitoses in several cells indicate active cell division.

2. *Proliferation Zone.* In this zone the cells are arranged in columns occupying more than half the total width of the cartilage plate. The cells are larger than the embryonic cells, are flat or wedge-shaped with their longest axis at right angles to the longitudinal axis of the columns. The nuclei are stained dark blue by haemotoxylin. The cell columns are separated by narrow strips of basophilic substance, while the space between the cells in each column contains very little matrix.

3. *Hypertrophic Cell Zone.* The cells in the half of the cartilage plate facing the diaphysis are larger and change shape. The flat cells initially assume a round or polygonal form, and finally may become elongated in the longitudinal direction of the columns. This zone contains even less matrix. The cells are vacuolated and the nuclei are stained slightly by haemotoxylin.

The cells in the area facing the diaphysis, or more correctly that part of the diaphysis closest to the epiphysis, often called the metaphysis, are removed regularly. Consequently the boundary of the metaphysis is sharply defined. The capillaries from the metaphysis invade the hypertrophic cartilage cells which open and disappear. The cartilaginous matrix between the cell columns remains and forms the core of the newly formed bone spiculae in the metaphysis where most of the bone salts are precipitated and deposited. The bone salts are initially precipitated in the epiphyseal plate in the vicinity of the last 2—3 series of hypertrophic

cartilage cells. This zone of cartilage can therefore be separated and is denoted as the *calcifying zone* of the cartilage.

The capillary buds invading the cartilage cells consists of blood vessels with accompanying mesenchymal fibroblasts. The connective tissues which are in contact with the cartilage plate are converted into osteoblasts. A thin acidophilic strip of osteoid tissue forms between the osteoblasts and the cartilage; becomes calcified and is called bone. The osteoblasts continue to form bone tissue, become completely submerged by it and are finally converted to osteocytes.

The same histological changes take place in the structure of the cartilage wherever there is endochondral bone formation. The cartilage cells hypertrophy, degenerate, and are invaded by vascularized connective tissue and replaced by primary bone marrow. These processes occur in the primary calcification of the diaphysis, in the epiphyseal head after the ossification centre is formed there, and in the epiphyseal plate. This process in the latter area is organized to serve as a growth apparatus for the longitudinal growth of the bone. Interstitial longitudinal growth of the diaphsyis does not take place. The factors involved in the growth of the epiphyseal cartilage are discussed in detail by P. Lacroix in "The Organization of Bones" from which most of the above description has been taken. In this monograph the essential facts are expressed as follows: "During the whole period of growth, the epiphyseal cartilage compensates by continuous regeneration for the destruction which it undergoes at its diaphyseal surface."

The epiphyseal cartilage plate is a temporary formation that disappears and is replaced by bone tissue when the bone ceases to grow in length. The epiphyseal plate closes at different times according to the type of bone and animal species. After the closing of the epiphyseal plates, the bone tissues from the epiphysis and diaphysis become continuous, but traces of the cartilage plate may remain for life as a calcified line, visible with X-rays and on histological investigation.

In this respect emphasis should be laid on the possibility that calcification of the cartilage and formation of bone are two distinct processes. On calcification of the cartilage, calcium phosphates are precipiated in the preformed matrix; on bone forma-

tion the osteoblasts form the ground substance which is immediately calcified. Bone formation therefore comprises both production of the organic ground matrix substance and precipitation of bone salts.

It still remains to be determined whether the same cellular processes are responsible for the precipitation of bone salts in both instances.

Chapter II.

Previous Investigations of Enzymatic and Metabolic Processes in the Epiphyseal Cartilage.

For almost a century it has been known that cartilage cells contain glycogen (Rouget 1859, Ranvier 1863). Marchand (1885) found that "the increase in size of the cartilage cells in the neighbourhood of the bone is due to the accumulation of glycogen or at least concides with it". Creighton (1896) maintained that "the cartilages which are destined to continue throughout life as cartilages have little or no glycogen in the foetal period, but those which later will ossify have plenty and it usually appears in the spots which afterwards become ossification centres". Pflüger (1907) determined quantitatively the glycogen content in the bones from a dog and found that diaphyseal bone contained 0.007 %, cartilage 0.2 % and marrow 0.3 % glycogen.

The glycogen in cartilage has been the object of more recent investigations employing histochemical technique (Sundberg 1924, Harris 1932, 1933, Gendre 1938). By means of improved histochemical methods, Follis and Berthrong (1949) investigated the distribution of glycogen in the epiphyseal cartilage and gave the following detailed description: "glycogen is found in increasing amounts as the cartilage cells mature. In young and most undifferentiated cells only a few small pink or reddish granules can be found. With multiplication of the cells and as they increase in size, more numerous and larger granules of glycogen appear, until, in the hypertrophic cells close to the cartilage-bone junction, the cytoplasm is filled with homogeneous reddish-

staining material. No glycogen can be found in the cell nuclei at any time. The most mature cells, that is those closest to the shaft and nearest the invading blood vessels, do not appear to contain glycogen."

Follis (1949) also investigated by histochemical methods the distribution of glycogen in rachitic cartilage. He found that "glycogen is present in rachitic cartilage only in the most recently matured hypertrophic cells. The broad zone of hypertrophic cells beneath is devoid of glycogen. This localization coincides with the area where inorganic elements deposit during the initial stages of healing —."

Glock (1940) investigated chemically the glycogen content in bones from young rats of different ages and found an increase during the first 10—13 days after birth followed by a rapid decrease.

Leynse (1952) analyzed glycogen content of rat tibiae at different stages after birth and found that it increased from 0.3 per cent in the first 6 days to about 0.5 per cent on the 12th day and then rapidly decreased.

Robison (1923) was the first to demonstrate the presence of phosphatase in the ossifying cartilage of young rats and rabbits and related this enzyme to the process of calcification.

Later an important group of chemical and histochemical observations have demonstrated that those regions of growing bone and teeth and of fracture calluses where deposits of salts are being organized, are rich in alkaline phosphatase (for references, see Jean Roche, 1950). Fell and Robison (1929 and 1930) showed a remarkable correlation between the histological differentiation of the embryonic femur *in vitro* and the appearance of phosphatase, but could not demonstrate any phosphatase in cartilage tissue which does not calcify *in vivo*. They assumed that phosphatase hydrolyzed the phosphate esters present and produced a local increase in the concentration of inorganic phosphate. The solubility product of calcium phosphate would consequently be exceeded and bone salts precipitated. Martland and Robison (1926) could, however, show only insignificant quantities of organic ester phosphate in plasma. Phosphate could also be demonstrated in other tissues that do not calcify *in vivo* under normal conditions (MacFarlane, Patterson and Robison 1934).

Robison evolved methods for the study of *in vitro* calcification which have since been extensively used in similar investigations. It could be shown that cartilage slices from rachitic animals calcify in solutions of inorganic salts saturated with calcium phosphate. Phosphate ester is required for calcification when the concentrations of calcium and inorganic phosphate are below the saturation values (Robison and Soames 1930).

In addition Robison and Rosenheim (1934) showed that low concentrations of fluoride and monoiodo-acetic acid inhibited *in vitro* calcification without affecting the activity of phosphatase. In consequence, they proposed the hypothesis that, besides the phosphatase mechanism, there was a second mechanism also of enzymatic nature. The second mechanism displayed, with respect to its inhibition by fluoride and iodo-acetic acid, certain similarities with the glycolytic processes in yeast and muscle. The results, however, did not exclude the possibilty that factors of a nature other than enzymatic e. g. surface forces in the colloidal matrix, also affect the precipitation of bone salts.

Hoffman et al. (1928) found that fresh cartilage slices slowly lost their glycogen content and that lactic acid was formed during incubation in phosphate buffer of pH 7.4. Dickens and Weil-Malherbe (1936) investigated by means of manometric techniques the oxygen uptake and lactic acid formation in slices of rib cartilage from rat in a bicarbonate-sodium chloride solution containing 0.2 % glucose. The values obtained for $Q_G^{N_2}$ and $Q_G^{O_2}$ were 1.85 and 1.22 respectively.

Bywaters (1937) used manometric methods to investigate the glycolytic activity of articular cartilage. $Q_G^{N_2}$ was found to be approximately 0.2 for normal articular cartilage with oxygen in the gas phase. These values refer to experiments where glucose was added to the incubation solution. Without the addition of glucose the formation of lactic acid was lower, about 0·09. In the two experiments reported, the Q-values for rib and knee cartilage of young rabbits were higher, 4.5 and 6.6 respectively.

The glycolytic activity per cell was found to be approximately the same for cartilage and other tissues. The oxygen uptake was too small to be measured, but the oxygen consumption of cartilage tissue was calculated to lie between 1/50 and 1/100 of that of other tissues.

Lutvak-Mann (1940) obtained similar results from articular cartilage of calf. She found that the glycogen content changed only slightly during protracted autolysis. Fresh articular cartilage contained 20—40 mg lactic acid per 100 g, practically all of which could be brought into solution by short extraction with water. Small amounts of lactic acid (2 g cartilage formed in 8 hours 0.38 mg of lactic acid at 37° C, pH 7.6) were formed on incubating cartilage slices, more being produced by the addition of 10 mg glucose to the incubating medium (4.18 mg under the same conditions). The oxygen uptake was very small when tested manometrically (1—2 µl per gram per hour). No cytochrome band could be spectroscopically detected in a thin layer of powdered cartilage.

Hills (1940) performed similar investigations of articular cartilage from horse and found low activity for both glycolysis and oxygen uptake. The glycolysis was inhibited by fluoride and iodoacetic acid.

Tulpule and Patwardhan (1954) employed manometric methods to investigate glycolysis and pyruvate oxidation in slices from rat epiphyseal cartilage. Contrary to all experience, they obtained high glycolytic activity in slices with glycogen and hexose phosphates as substrates, substances that cannot penetrate cell membranes (Rosenberg and Wilbrandt 1952). A possible explanation of the observed activity may be that the adenosine triphosphate, which was added in all the experiments to the incubation medium, was hydrolyzed by the phosphatase present in epiphyseal cartilage, with the subsequent liberation of two extra acidic groups. The production of acid may be explained by the addition of ATP in almost all the experiments. Tulpule and Patwardhan do not provide any information that may serve in evaluating this possibility. It may be valuable in this respect to recall the following: "Doch darf nicht vergessen werden, dass die Druckmethode eine unspezifische Methode ist, und dass die Natur derjenigen Stoffe die den Druck hervorbringen, durch chemische Kontrollmethoden festzustellen ist" (Minami 1923).

In addition Tulpule and Patwardhan found that, compared with slices from controls treated with vitamin D, there was less pyruvate oxidation in the epiphyseal cartilage from rachitic animals. The difference, in this instance, can be due to the different

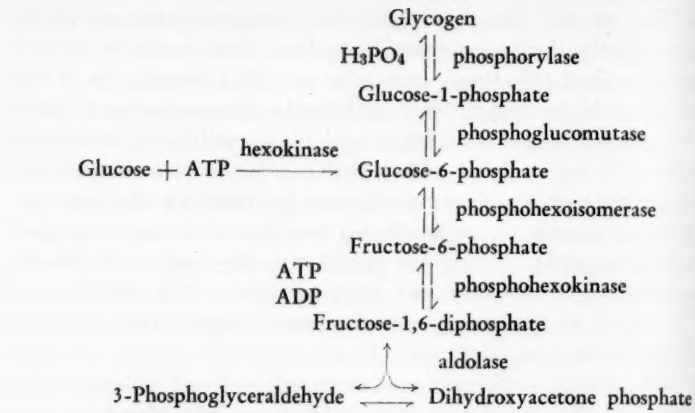

Figure 1.

Pathway of anaerobic glycolysis.
(Embden-Meyerhof Scheme.)

qualities of tissue compared. The hypertrophic epiphysis of rachitic animals consists mainly of hypertropic cells and osteoid substance, as opposed to epiphyseal cartilage in normal animals. It is difficult to conceive how a comparison of such heterogeneous material can lead to any definite conclusions.

A number of investigations confirm the presence of glycolytic processes in cartilage tissue. The activity is dependent on the material used and observed differences in activity are probably due to different cell contents.

In more recent years considerable progress has been made towards elucidating the intermediary carbohydrate metabolism of cells. Each single stage in the decomposition of glucose or glycogen to lactic acid is well known. The intermediary metabolites have been isolated and a number of the enzymes involved have been prepared in crystalline form. Consequently we can now follow the carbon chain of glucose through a series of reactions, each catalyzed by a specific enzyme (Fig. 1).

These reactions result in the formation of lactic acid and the transformation of the energy liberated during the process via the pyrophosphate bond in adenosine triphosphate.

The existence of a number of these enzymes in cartilage have been demonstrated by suitable methods. By means of chemical methods Gutman and Gutman (1941) found phosphorylase in epiphyseal cartilage from young rats and rabbits. Albaum et al. (1952) succeeded by optical methods in demonstrating the existence of several glycolytic enzymes (aldolase, triosephosphate dehydrogenase, enolase and lactic acid dehydrogenase) in extracts from rachitic rat bones. The presence of several phosphate esters in the glycolytic cycle was successfully confirmed by means of radioautography of paper chromatograms of trichloroacetic acid extracts of rat tibia. (24 hours in advance the rats had been administered intraperitoneally a large dose of radioactive phosphate.) Albaum et al. (1952 a) revealed by means of very sensitive methods adenosine triphosphate in trichloroacetic acid extracts of epiphyseal cartilage from rats.

The phosphate esters, however, exist only in very low concentrations, too low to be determined by ordinary chemical methods (Lutvak-Mann 1940, Cartier 1951).

Chapter III.

Previous Investigations of and Hypotheses on the Mechanism of Calcification.

The chemical structure of the inorganic components of bone has been known for a long time. The structure and size of bone salt crystals have been discussed fully in recent years (Armstrong 1950, Hendrichs 1952, Robinson 1951). Hodge (1951) discussed, in the light of experiments with inorganic model systems, the manner of precipitation of bone salts. Apparently a complete explanation of the precipitation of bone salts cannot be given on the basis of purely physico-chemical considerations.

The stability of the skeleton is primarily the result of the mineral balance — in living organisms the quantity of bone salt depends on the balance between intake and output of calcium and phosphate. In the second place the stability of the skeleton is a function of a dynamic equilibrium between matrix formation and dissolution. During growth new formation dominates, but in certain diseases dissolution can be dominant. The simultaneous formation (or decomposition) of matrix and the precipitation (or dissolution) of bone salts are suggestive of an intimate relationship between the two components. The obvious assumption is therefore that the organic intercellular substance in one way or another can bind or adsorb bone salts. This hypothesis formed the basis of a series of experimental investigations performed by Freudenberg and György (1921). By incubating rib or sternum cartilage from calves or humans they could show that cartilage adsorbed calcium ions. A calcium saturated cartilage could bind phosphate ions, while a fresh, unsaturated cartilage could not. Boyd and Neuman (1951) have recently confirmed and extended these observations. The capacity of rib cartilage from calf to bind certain cations (sodium, calcium and barium) correlated well with the sulphate content of the cartilage. This finding suggests that chondroitin sulphate is the principal binding agent. Phosphate was only adsorbed by cartilage with a high calcium content. Neuman (1950) and Neuman et al. (1951) showed that powdered

bone preparations are capable of crystal growth when immersed in a synthetic ultrafiltrate of plasma. The presence of glycerophosphate in the ultrafiltrate inhibited the formation of crystals.

Ester phosphate is strongly adsorbed by bone ash. In addition ester phophate inhibited in vitro calcification of rachitic cartilage slices when the phosphate activity was inhibited by the addition of corrosive sublimate (mercuric chloride). On the basis of these experiments, Neuman suggests the possibility of ester phosphate having an inhibitive effect on calcification. The phosphate esters that are adsorbed will interfere with the succeeding deposition of ions on preformed crystals surfaces. Accordingly, at least part of the function of phosphatase lies in removing the inhibitive influence of ester phosphate.

On the basis of histochemical investigations, Siffert (1951) has proposed that phosphatase is related to the preosseous cell metabolism and to the formation of a matrix capable of calcifying, rather than to the calcifying mechanism itself. He has pointed out the fact that matrix formation, which is always accompanied by phosphatase activity, can take place without calcification. Calcification can proceed at a later stage in the absence of the enzyme.

The effect of selective phosphatase inhibitors on in vitro calcification of rachitic cartilage slices has been investigated (Gutman and Yu 1951, Hiatt and Shorr 1951). It was shown that calcification was inhibited by beryllium (1×10^{-4}M) and by histidine (1×10^{-2}M) when phosphate esters formed the only phosphate source. Considerably higher concentrations of these inhibitors were necessary when the incubation medium contained inorganic phosphate.

As already found by Robison, *in vitro* calcification of rachitic cartilage slices is inhibited by substances having a selective inhibitive effect on certain stages of glycosis. When the calcification medium contains inorganic phosphate; the inhibition is cancelled by phosphate ester (glycerophosphate). As early as 1930, Harris drew the analogy with the glycolytic processes in yeast and muscle and suggested that the decomposition of glycogen in cartilage might produce hexose phosphates as substrate for the phosphatase. No experimental support for this hypothesis was available, however, as at that time glycogen was belived to

be decomposed by the action of amylase. J. Roche (1947) proposed a similar mechanism and suggested that glucose-1-phosphate which is formed in the phosphorylase reaction from blood phosphate ions and bone glycogen, is hydrolyzed by bone phosphatase resulting in a high local concentration of inorganic phosphate.

Gutman and Yu (1949, 1950) have made a more systematic study of the effect of selective inhibitors in the glycolytic cycle on the *in vitro* calcification. They showed that phlorrizine inhibits calcification with inorganic phosphate and that the calcifiability can be restored by glucose-1-phosphate or by other phosphate esters in the glycolytic cycle. In the case of iodo-acetic acid poisoning the calcifiability can be restored by adding 3-phosphoglyceric acid or glycerophosphate. Fluoride inhibits calcification with inorganic phosphate and with all the other phosphate esters tested with the exception of glycerophosphate. On the basis of these experiments Gutman assumes that glycolysis must at least reach the phosphopyruvic stage before it can contribute to the mechanism of calcification.

Marks and Shorr (1950) confirmed these experimental results by a different technique. They evolved a method to demonstrate glycogen in surviving slices. The glycogen content of hypertropic cells was considerably diminished by incubation in a salt solution free of calcium and phosphate or by treatment with saliva. When these pretreated slices were incubated in a calcifying medium with inorganic phosphate, the deposit of calcium was considerably smaller than in the control slices. If the incubating medium contained glucose-1-phosphate, the pretreated and control slices deposited equal amounts.

Taken as a whole, it is evident that calcification in epiphyseal cartilage is a considerably more complicated process than a simple precipitation of calcium phosphate. There are many factors implying that a series of enzyme systems and metabolic processes are implicated. Biochemical and histochemical observations suggest that phosphorylative glycolysis is an integral part of the system. Further details of the action of phosphatase and the part played by glycolysis and their exact role in the system have not been reported in previous investigations. Whether the action of phosphatase lies mainly in the formation of the organic ground

substance or more directly in the calcification mechanism is still unknown. If the latter alternative is correct, it still remains to decide whether its task is to split ester phosphate before its precipitation in matrix, or whether ester phosphate is first adsorbed as such on to the matrix and subsequently split. The position of glycolysis in the calcification mechanism is also unclear and this may be illustrated by the following quotation: "Whether glycogenolysis is essential in order to provide phosphorus in some special form or concentration or place, or whether required energy is made available, is not clear." (Gutman and Yu 1949.)

PART II. EXPERIMENTAL

Chapter IV.

The growth of tibia.

The first investigations were undertaken to establish an expression for the rate of the longitudinal growth of tibia and to determine the contributions of the proximal and distal growth zones to the total growth.

A. *Materials and methods.*

Weanling rats from a strain bred in the Institute for several years were used. The mothers were fed the standard stock diet. The total length of tibia was determined by direct measurement after dissection. The contribution of the two growth zones to the total growth was determined as follows: A thin metal thread was inserted in the central part of the tibia of one week old rats. The metal thread, supported by a straight sutur needle was led percutaneously through the tibia and back to where the thread was knotted. The length was measured on roentgenograms taken the same day and then at intervals on the same rats.

For the histological examinations the tibiae were fixed in formalin, decalcified in dilute nitric acid, embedded in paraffin, cut in slices 10 micron thick and stained with haematoxylin-eosin. The size of the cells was determined by means of a calibrated eyepiece micrometer.

B. *Results.*

The results of the direct measurements of the length of tibia at different ages are illustrated in Figure 2. After 7 days the tibia is 10 mm long and increases linearly to 20 mm in the following 14

days. The longitudinal growth then proceeds more slowly. The increase in the second week after birth is 5,8 mm, in the third 4,2 mm and in the fourth 1,8 mm.

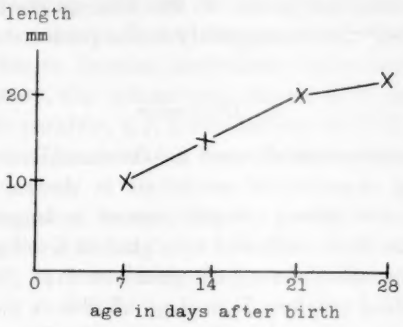

Figure 2.
Length of rat tibia during the first four weeks after birth.

The results of the roentgenogram measurements to determine the contributions of the proximal and the distal epiphysis to the total growth are reported in *Table 1*.

Table 1.

Determination of the growth rate of proximal and distal epiphyseal cartilage of rat tibia. Procedure described in text. Length of proximal part is the distance from metal ring to knee joint and of distal part from metal ring to ankle joint.

Rat no.	Age	Total length	Length of		Increase of prox. part. % of total length
			prox. part	dist. part	
	days	mm	mm	mm	
1	7	10.8	5.5	5.3	
2	7	10.3	4.9	5.4	
1	17	16.8	9.5	7.3	67
2	17	17.3	9.3	8.0	63
1	24	20.4	11.5	8.9	63
2	24	21.0	11.2	9.8	59
1	31	22.3	13.0	9.3	65
2	31	23.0	13.2	9.8	65

Approximately ⅔ of the increase in length is contributed by the proximal epiphysis. In addition it is seen from the table that tibia grows only very slowly at the distal end during the last week of the observed period — the increase during this week must be ascribed almost completely to the proximal epiphysis.

C. *Discussion.*

The bone most commonly used for the measurement of growth under varying experimental conditions in the rat is the tibia. The growth curve shows a rapid increase in length during the first weeks after birth, followed by a gradual levelling off. Maximal length is attained during the first 100 days (Simpson et al. 1950). The distal epiphyseal cartilage of tibia is closed after 90 days, whereas the proximal epiphyseal plate is still open in senility. Abrupt narrowing of the plate has been noted in early adult life at the time of closure of the majority of epiphysis. Such persisting epiphyseal plates become dormant, sealed by bony plates from the marrow. The abrupt narrowing coincides with the levelling off of the growth curve. Even if the proximal epiphysis remains open in old age, it is obvious that it does not function under physiological conditions.

Estimation of the life span of cells in the epiphyseal plate.

Growth of the bone tissue takes the form of a compensation for the cartilage tissue which is destroyed on the diaphyseal surface of the cartilage plate. The cartilage compensates continuously for this destruction with new cartilage tissue. The width of the cartilage plate is unchanged during the whole of the rapid phase of the growth period. This fact signifies that every hypertropic cell which is destroyed in the calcifying zone is replaced by a new cell in the cell division zone.

The contribution of the proximal epiphyseal plate to the longitudinal growth per time unit was found by measuring the growth. When the diameter of the hypertropic cartilage cells as well as the number in each cell column in the cartilage plate are measured, it is a simple matter to determine the lifespan of the individual cell from the division to the destruction zone.

The average diameter of the hypertropic cartilage cells was measured on standard haemotoxylin-eosin stained slices and found to be approximately 30 μ. In every cell column there are 5—10 hypertropic cartilage cells and 20—30 cells in the whole column.

No allowance was made for changes in the size of the hypertropic cells due to fixation, embedding and staining. According to Baker (1950), the volume may shrink 40 % on fixation and embedding in paraffin, i. e. a contraction of 15 % in the length of a cubic side. This shrinking was not taken into account in the present calculations.

The measurements showed that the growth was approximately linear during the 2nd and 3rd weeks after birth and the increase in length was 10 mm during this period. The epiphyseal plate contributed ⅔ or 6.6 mm, i. e. 0.47 mm/day or 0.02 mm/hour if continuous growth is assumed.

Calculation.

A hypertropic cell, with a diameter of 30 μ and a longitudinal growth of 20 μ per hour, takes 1½ hours to pass from the border of the diaphysis to destruction. The cartilage must have grown correspondingly in the same period and a new cell must have reached the top of the cell column from the division zone. As there are 20—30 cells in each column, each cell will require 30—45 hours for its development from the division to the border zone of the bone tissue. The best part of its lifetime will be spent in the proliferation zone. The hypertropic cell zone, where there are 5—10 cells, will be traversed in the course of 7½—15 hours, and the calcifying zone of the cartilage corresponding to the last 2—3 cells, in the course of 3—4½ hours.

A constant cartilage width signifies an equilibrium between formation of new cells in the division zone and destruction of old cells in the calcifying zone. If the rate of formation is greater than the rate of destruction, the width of the cartilage diminishes; the converse is also true. The width of the cartilage diminishes in almost all cases of nutritional deficiency, and is most pronounced in caloric undernourishment. It is also noticed in partital deficiency e. g. amino acid deficiency. In this case the reduction in width may be adequately explained in terms of a

decrease in formation of new cells. Increase in cartilage width is noted along with increase in growth following administration of growth hormone. This effect of growth hormone on the epiphyseal plate is characteristic and is utilized for quantitative hormone determination (Greenspan et al. 1949). In rickets, the increased width of the cartilage results from a considerable delay in cell destruction with an unchanged formation and development of the cartilage cells.

Chapter V.

The Water, Ash, Lactic acid and Glycogen Content of Tibia in young Rats.

The quantitative ratio between cartilage and bone tissue changes during growth, and the cartilage structure also changes. During the first week after birth the whole of the epiphyseal head is comprised of cartilage, but towards the 10th to the 12th day the bone centre in the proximal epiphysis of tibia and distal epiphysis of femur assumes its initial form. Before the bone centre is formed, the cartilage undergoes the same changes as the calcifying cartilage when the cells hypertrophy before the invasion of the blood vessels. The following analysis were performed to ascertain whether these morphological changes are reflected in the chemical composition.

A. *Methods.*

For chemical analysis the rats were killed by a blow on the head and bled. The tibia were dissected and all soft tissue carefully removed. The fresh bone were weighed as soon as possible after dissection. The dry weight was determined after drying at 105° C to constant weight. The ash content was determined by heating at 700° C for approximately 3 hours in platinum crucibles.

Lactic acid determination. The method of Barker and Summerson (1941) as modified by LePage (1945) was used. The bones were extracted with 10 % trichloroacetic acid. Blanks and standards (prepared from zinc lactate) were run in parallel for each series.

Glycogen analysis. Two methods were employed: Walaas' micro-method (1950) and Seifter's anthrone method (1950).

a) Walaas' method was slightly modified to suit the special circumstances connected with bone tissue. The bones (or piece of tissue) were placed in small centrifuge tubes containing 0.1 ml 30 % KOH for each 50 mg tissue weight. After hydrolysis in stoppered vessels on a water bath at 100° C for 30 minutes and subsequent cooling, 0.05 ml 2 % sodium sulphate solution was added, followed by an amount of absolute alcohol such as to give a final alcohol concentration of 65 %. With larger bones having a high ash content the alkali hydrolysate was centrifuged to remove insoluble salts, and an aliquot of the supernatant was taken for precipitation of the glycogen. After settling for at least 3 hours, the glycogen containing sediment was reprecipitated, then washed with 75 % alcohol, and the traces of alcohol carefully evaporated off. The dry sediment was then hydrolyzed with 1 N sulphuric acid for 3 hours. After neutralization and dilution to a known volume, an aliquot was taken for glucose determination by Nelson's colorimetric method (Nelson 1944).

b) Seifter's method. Hydrolysis with alkali and precipitation with alcohol were performed according to Walaas' method. After a final washing of the glycogen-containing sediment, the latter was dissolved in a known quantity of distilled water. An aliquot was added to enough water to give a total volume of 2.5 ml. To this was added 5 ml anthrone reagent and the contents shaken well. The vessels were placed in a water bath at 100° C for 10 minutes and cooled. The optical density were measured on a spectrophotometer at 620 mμ. Control samples containing 20 and 40 μg glucose were included in each series.

As Walaas' method for glycogen is based on the conversion of glycogen to glucose, the reducing sugar may possibly originate from other components of the cartilage e. g. chondroitin sulphate. This possibility was ruled out in the following way: Two samples of epiphyseal cartilage were cut from the proximal end of the tibia and the distal end of the femur of 8 day old rats. The samples were digested with alkali, precipitated with alcohol and hydrolyzed with acid in the usual way. The solutions were neutralized and made up to 2 ml. From these solutions aliquots were taken for glucose determinations, performed before and after

fermentation. For direct glucose determination 0·5 ml aliquot was mixed with 1.5 ml water, 0.5 ml barium hydroxide solution and 0.5 ml zinc sulphate solution. After centrifugation 1 ml of the supernatant was taken for glucose determination by Nelson's method. To another aliquot (0.5 ml) of the same solution 1.5 ml of a 10 % suspension of washed yeast cells (bakers yeast) was added and incubated for 20 minutes at room temperature. The same amounts of barium hydroxide and zinc sulphate were then added and a 1 ml sample taken for a glucose determination. A blank without cartilage hydrolysate was treated in the same way to determine any possible reduction value due to yeast cells. This blank value was negligible. The results are illustrated in Table 2.

Table 2.

Fermentation of glycogen isolated from the epiphyses of 8 day old rats. Analytical procedure described in text.

Sample no.	Amount of cartilage	Glucose content of aliquot		Glycogen
		direct	fermented	
	mg	µg	µg	mg/g
1	66.6	56	2	9.7
2	66.2	57	2	9.9

The Nelson method is relatively specific and does not give colour with glucosamine or glucuronic acid, the main components of chondroitin sulphate. The fermentation with yeast is selective and only glucose, fructose and mannose are fermented. There is very little possibility that hexoses other than glucose will be found in significant quantities. It can be concluded that the method used, does give correctly the glycogen content of the cartilage.

B. *Results.*

The results of these investigations are presented in Tables 3—7. The body weight varied slightly from litter to litter in the different age groups. The cause of this variation lies partly in the size of the litter — each young rat in a small litter gets more milk from the mother — and partly in inaccurate knowledge of the time of birth.

Table 3.
Growth of tibia during the first 4 weeks after birth.

Age	Body weight	Weight of tibia		Dry weight in per cent of wet weight	Length
		wet	dry		
days	g	mg	mg	%	mm
7	12.1	25.4 ± 0.65	7.6 ± 0.16	30	10.0 ± 0.12
14	26.7	61.4 ± 2.5	24.3 ± 0.4	41	15.8 ± 0.16
21	38.2	111.0 ± 0.42	46.3 ± 1.5	42	20.0 ± 0.19
28	47.5	139.7 ± 1.7	65.4 ± 0.7	47	21.8 ± 0.13

Each value represents the mean value obtained by analysing the tibiae from 5 rats. Standard deviation calculated according to: $\sqrt{\frac{\Sigma \triangle^2}{n(n-1)}}$

Table 4.
Ash content of tibia during the first 4 weeks after birth.

Age	Body weight	Weight of tibia		Ash weight	Ash in per cent of dry weight
		wet	dry		
days	g	mg	mg	mg	%
7	—	32.0 ± 0.6	8.2 ± 0.13	2.3	28
14	27.6 ± 0.56	79.2 ± 2.5	27.1 ± 0.76	9.7 ± 0.3	36
21	37.5 ± 0.66	118.7 ± 2.3	44.0 ± 1.1	16.5 ± 0.5	38
28	55.0 ± 0.7	167.8 ± 3.8	74.3 ± 1.6	33.0 ± 0.5	44

Each value represents the mean value obtained by analysing the tibiae from 8 rats.

The tibial weight varied according to the different body weights of the litters at various ages. The tibial weight (both wet and dry) increases approximately linearly with age (Tables 3 and 4). The ash conten. increases more rapidly in the fourth week after birth than in the preceding weeks.

The lactic acid content (Tables 5 and 6) increases steadily with age and the "concentration" of lactic acid in the tissue remains round about 0.6 mg/g wet weight at all ages.

The glycogen content (Table 7) increases in the first three weeks, followed by a decrease. The glycogen content in 2 and 4 week old rats is approximately the same.

Table 5.

Lactic acid content of the tibia during the first 4 weeks after birth.

Age	Wet weight of tibia	Total lactic acid content	Lactic acid
days	mg	μg	mg/g
7	24.7 ± 0.8	15.2 ± 0.8	0.62
14	48.5 ± 1.5	33.6 ± 2.5	0.70
21	105.0 ± 3.0	60.0 ± 3.1	0.57
28	124.3 ± 3.2	78.2 ± 4.1	0.63

Each value represents the mean value obtained by analysing the tibiae from 5 rats.

Table 6.

Lactic acid content (see table 5) calculated from the values given in table 3 for the dry matter and water content.

Age	Wet weight	Calculated dry weight	Calculated weight of water	Lactic acid content calculated on basis of	
				dry weight	water
days	mg	mg	mg	mg/g	mg/g
7	24.7	7.4	17.3	2.06	0.88
14	48.5	19.4	28.1	1.73	1.20
21	105.0	44.0	61.0	1.36	0.98
28	124.3	58.0	66.0	1.35	1.19

Table 7.

Glycogen content of the tibia during the first 4 weeks after birth.

Age	Body weight	Wet weight of tibia	Total glycogen content	Glycogen content per unit weight
days	g	mg	μg	mg/g
7	15.0	37.8 ± 1.4	160 ± 4.2	4.24
14	27.7	83.4 ± 2.3	350 ± 17	4.21
21	42.0	132.0 ± 4.5	446 ± 29	3.40
28	57.2	166.0 ± 4.0	322 ± 13	1.94

Each value represents the mean value obtained by analysing the tibia from 7 rats.

C. *Discussion.*

The results for the tibial content of dry substance and ash should be considered in relation to the morphological changes taking place during growth. The increasing content of dry substance and ash indicates that cartilage tissue having a high water and a low ash content is replaced by bone tissue.

The total lactic acid content increases steadily with tibial weight and, calculated from the wet weight, has a value round about 0.6—0.7 mg/g. There is often doubt about how best to express the tissue content of the different metabolites. The usual expression is in terms of dry weight. This is not so important when the tissue has a relatively constant water content, but it matters when the water content varies. Calculations of the lactic acid content at different ages, partly expressed in terms of dry weight, partly in terms of the water content of the tibia, are presented in Table 6. Dry weight and water content are here calculated in terms of values given in Table 3. The lactic acid content expressed as mg per gram dry weight, decreases with increasing age. The values are in good agreement with those obtained from the analysis of sheep bones. (Cartier 1951 a.) The lactic acid concentration in the "tibial water" varies from 0.88 to 1.20 mg/g and shows no relation to the size of the tibia. A combination of biological variability and analytic error are considered to be the causes of the variation. Lactic acid diffuses easily through cells and tissues and any local increase in the individual cell zones can hardly be expected.

Compared to other tissues, the tibia has a high content of lactic acid. Barker and Summerson (1941) found 25 mg% in brain, 10—35 mg % in heart and 150—200 mg % for the entire rat carcass; Lutvak-Man (1941) found 20—40 mg per 100 g wet weight in articular cartilage; Albaum et al. (1946) found 20—40 mg % in rat brain.

It is possible that special conditions in the bones may cause a higher lactic acid content than in other organs with more active metabolism and better blood circulation. Barker and Summerson's observation of the high lactic acid content in the entire carcass may partly be explained by the high lactic acid content of the skeleton.

The total glycogen content of the tibia reaches a peak at the end of the 3rd week, then decreases. Expressed as mg glycogen per gram wet weight, the content remains constant (4.2) during the first two weeks, decreases to 3.4 in the third and to 1.9 in the fourth. These results are at variance with Glock's findings, namely that the glycogen content in young rat bones increased

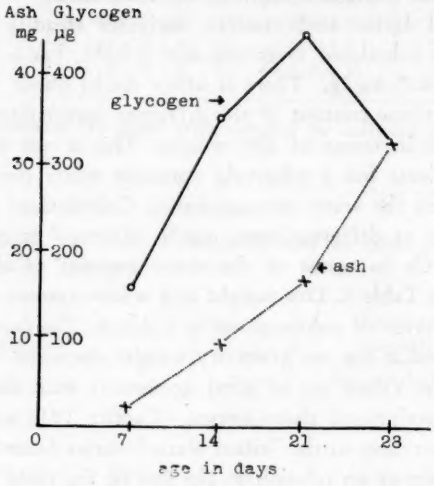

Figure 3.

The total ash and glycogen content of rat tibia during the first 28 days after birth.

from 4.0 mg/g (3 days) to 5.28 mg/g (6 days) reaching a maximum of 7.4 mg/g (10 days). A rapid decrease to 2.4 mg/g (17 days) and 0.8 mg/g (26 days) followed. Glock (1940) used the long bones of right and left fore- and hind limbs and it is possible that there are considerable differences in the glycogen content of the individual bones during growth.

The curves for the glycogen and ash content of the tibia at different ages are drawn in Figure 3. It is obvious that the highest rate of increase in ash content which occur during the 3rd and 4th weeks coincides with glycogen reduction. This fact supports the histological observations in favour of a relationship between glycogen and the calcifying processes.

D. *The Distribution of Glycogen in Tibia.*

The histochemical studies of previous workers on the distribution of glycogen in the various sections of tibia were supplemented with quantitative chemical methods. The former show that the epiphyseal head consists exclusively of cartilage during the first 10—12 days after birth. At this stage the bone center in the epiphyseal head begins to develop and increases rapidly in size during the following weeks. The morphological changes are typical of those taking place during all endochondral bone formation (previously described). Quantitative determination of the glycogen in the entire epiphyseal head at different ages could therefore be expected to offer further information about the relation between glycogen and the calcifying process.

Tibia from a 3 week old rat was divided into proximal epiphysis, diaphysis and distal epiphysis and each part was analyzed separately by the anthrone method. The results are presented in Table 8.

Table 8.

Glycogen content in different parts of the tibia.

	Wet weight	Glycogen	
	mg	μg	mg/g
Proximal epiphysis	28.0	256	9.14
Diaphysis	55.3	36	0.65
Distal epiphysis	20.5	64	3.12

About ¾ of the total glycogen content of the tibia is localized in the proximal epiphyseal head. The distal epiphysis is much smaller than the proximal and contains considerably less glycogen, whether expressed absolutely or in mg/g tissue. The dividing line is not sharply defined on account of its irregular shape. The weight of the distal epiphysis is surprisingly large and it is possible that a part of the diaphyseal bone tissue has been included.

In order to limit the number of animals used but at the same time secure adequate material, the proximal epiphysis of the tibia

and the distal epiphysis of the femur were used. Histological examination show that the bone centre develops simultaneously in those two epiphyses. The results are given in Table 9.

Table 9.

Glycogen content of proximal tibial and distal femoral epiphysis of rats during the first 4 weeks after birth.

Age	Amount of tissue	Glycogen	
days	mg	µg	mg/g
7	52.2	535	10.2
14	45.9	549	12.0
21	73.3	758	10.3
28	124.3	1017	8.2

The total glycogen content increases more or less steadily during the first 28 days after birth, whereas the concentration reaches a peak after 14 days.

The morphological development between the 7th and 11th day is characterized by the evolution of the small celled cartilage in the central areas of the head into hypertropic cells. On the 11th day these are destroyed and replaced by primary bone marrow. The bone centre increases thereafter in size up to the 28th day. During this period the bone centre is surrounded by several layers of hypertropic cartilage cells. This development is accompanied by a steady increase in the total glycogen content. At the same time the amount of bone tissue in the centre increases to such an extent that the glycogen concentration in the tissue remains constant.

E. *The Distribution of Glycogen in the Epiphyseal Plate.*

From a morphological point of view, the epiphyseal plate consists of two main zones: the proliferation zone and the hypertropic cell zone, each comprising about one half of the thickness of the cartilage. A few simple experiments were performed to determine the glycogen content of each zone. Slices were cut with a razor blade perpendicular to the longitudinal axis of the

bone through the epiphyseal head of isolated tibiae of 14 day old rats. The slices proximal to the epiphyseal plate were rejected. The epiphyseal plate was divided as best possible in two equally thick slices, one proximal and one distal. The slices were weighed on a torsion balance and the glycogen content determined directly by the anthrone method. Precipitation by alcohol was not included when only small glycogen quantities were present. The results are shown in Table 10.

Table 10.

Distribution of glycogen in the epiphyseal plate from the proximal end of tibia of 14 day old rats.

Sample no.	Part of epiphyseal plate	Weight of tissue	Glycogen	
		mg	µg	mg/g
1	Proximal	2.3	30	13.0
	Distal	1.6	14.6	9.1
2	Proximal	2.9	29.3	6.6
	Distal	2.2	13.0	5.9
3	Proximal	2.7	25.3	9.4
	Distal	3.0	21.3	7.1

There is slightly more glycogen in the proximal half of the epiphyseal plate. On the assumption that the slices really represent the defined areas, this observation implies that the proliferation zone contains more glycogen per unit weight than the hypertropic zone. However, the proliferation zone contains considerably more cells per unit weight than the hypertropic zone. Therefore each cell in the hypertropic zone contains more glycogen. Histochemical observations showed that the last 2 or 3 series of hypertropic cells did not contain glycogen, consequently there are even fewer cells to contain the glycogen in this zone.

The technique employed in these analysis, however, is imperfect. Histological control of localization combined with cell counts, accurate weighing and accurate chemical analysis are all required to establish precise values for the glycogen distribution. The glycogen content of the individual cells in the different slices could then be determined. Such a procedure, however,

would imply a considerable amount of work, e. g. the development of the necessary histochemical micromethods. In the present work the simpler procedure was chosen to give a rough estimate and the results support the histochemical observations.

Chapter VI.

Lactic Acid Formation in Slices from Epiphyseal Cartilage.

The glycolytic activity of slices of cartilage has been studied by other workers. In the present work, however, it was found necessary to obtain quantitative expressions for the activity to make possible a correlation with the growth rate and the life span of the cartilage cells. The purpose of these investigations was also to get more detailed information about the glycolytic processes under varied experimental conditions.

A. *Procedure and Methods.*

Both tibiae were dissected and all soft parts carefully removed. A razorblade was used for slicing. The epiphyseal head was cut through the distal part of the bone centre, and the epiphyseal plate was cut into thin slices perpendicular to the longitudinal axis of the bone. The epiphyseal plate is rather flat in these young animals (about 3 weeks old) and slices weighing from 11—17 mg were successfully obtained. The epiphyseal cartilages of some tibiae are cup-shaped, and to ensure that no adjacent bone tissue was included, less cartilage had to suffice. The weight of the slices therefore varied slightly. The slices were quickly weighed and transferred to incubation vessels in an ice bath. The incubation vessels contained 0.2 ml of a buffer salt mixture; pH 7.4, 0.123 M NaCl, 0.005 M KCl, 0.003 M $MgSO_4$ (Cohen 1946). The small fluid volume was placed in the side arm of a standard Warburg vessel. Unless otherwise stated, the incubation time was 1 hour, the temperature 37° C and the rate of shaking 100 oscillations per minute. The slices from one tibia were incubated, while the slices from the other tibia of the same rat served as controls.

The control slices were placed in a corresponding quantity of incubating fluid (0.2 ml) in small reagent vessels which were kept in an ice bath until analyzed.

Known quantities of the substances whose effect should be studied were added to the incubation medium.

At the end of the experiment the Warburg vessels were placed in an ice bath. 50 µl of the incubating fluid was pipetted out for glucose determination by the Nelson method and 100 µl for lactic acid analysis according to Barker and Summerson.

B. *Results.*

The results from a typical incubation experiment are presented in Table 11. Compared to the control which remained constant, the glucose content of the medium diminished during incubation. The difference represents the glucose uptake of the slices and is calculated as mg glucose lost per gram cartilage.

Table 11.

Glucose uptake and lactic acid formation in slices of epiphyseal plate from 14 day old rats. Standard reaction mixture with 150 mg % glucose; pH 7.4; temperature 37° C; incubated for 60 minutes.

Animal no.	Weight of slices	Total amount in reaction mixture		Glucose used	Lactic acid formed
		glucose	lactic acid		
	mg	µg	µg	mg/g	mg/g
1	10.5 i	276	25.8	2.28	2.02
	11.2 c	300	5.0		
2	14.5 i	270	39.4	2.07	2.40
	17.0 c	300	5.4		
3	13.7 i	269	31.8	2.26	2.00
	16.5 c	300	5.5		
4	12.0 i	267	27.2	2.74	1.78
	16.4 c	300	7.9		
5	13.6 i	254	31.8	3.38	1.92
	11.9 c	300	5.0		

i: incubated slices.
c: control slices.

Table 12.

Glucose uptake and lactic acid formation in slices of epiphyseal cartilage from rats (body weight 45—50 g). Varied times of incubation. Experimental conditions as described in Table 11.

Time of incubation	Weight of slices	Total amount in reaction mixture		Glucose used	Lactic acid formed
		Glucose	Lactic acid		
min.	mg	μg	μg	mg/g	mg/g
30	11.0 i	295	26.6	0.45	1.61
	11.5 c	300	9.3		
30	14.6 i	277	25.5	1.58	0.87
	11.9 c	300	10.5		
60	Mean values from table 11.			2.55	2.02
120	11.7 i	256	52.5	3.76	3.59
	13.5 c	300	12.3		
120	13.8 i	245	53.5	4.00	3.11
	16.2 c	300	12.4		
180	15.0 i	213	81.6	5.80	4.35
	10.8 c	300	12.0		
180	11.6 i	216	73.5	7.25	5.42
	15.2 c	300	14.0		

i: incubated slices.
c: control slices.

Table 13.

Lactic acid formation with different amount of cartilage. The results are collected from different experiments under standard conditions (pH 7.4; glucose concentration 150 mg %; temperature 37° C; incubation time 60 minutes). The lactic acid formation is corrected for the lactic acid content of control slices.

Weight of cartilage	Lactic acid formed	
mg	μg	mg/g
8.2	16	1.95
10.5	21	2.00
11.2	23	2.05
13.7	27	1.97
14.5	35	2.41
16.8	38	2.13
17.8	41	2.30
19.5	42	2.16

The lactic acid content of the medium increases during incubation. Freshly sliced epiphyseal cartilage contains lactic acid, practically all of which is brought into solution by a short aqueous extraction of the slices. The lactic acid content of the control slices ranges from 0.5 to 1.0 mg/g (wet weight) and is consistent with the values obtained from analysis of the entire tibia at different ages. This fact supports the assumption that the lactic acid is distributed evenly throughout the entire bone, without any appreciable differences in concentration. The lactic acid formed in the slices during incubation diffuses into the solution. The lactic acid formation has been corrected for the lactic acid content of the control slices and has been calculated as mg/g.

The results for both glucose uptake and lactic acid formation show considerable variation. Variations of approximately the same size has been previously found by Bywaters (1937) in manometric investigations on cartilage tissue. The results have therefore to be cautiously evaluated.

Glucose consumption and lactic acid formation are proportional to the incubation time (Table 12); the lactic acid formed is also proportional to the weight of the slices in the vessel (Table 13).

Table 14.

Effect of pH on glucose uptake and lactic acid formation in slices of epiphyseal cartilage. Standard conditions, 150 mg % glucose, temperature 37° C, incubation time 60 minutes.

Rat no.	pH	Weight of slices	Glucose used		Lactic acid formed	
		mg	μg	mg/g	μg	mg/g
1	7.4	15.8	64	4.05	34	2.15
2	7.4	14.3	36	2.52	31	2.15
3	6.36	12.4	0	0	17	1.37
4	6.36	13.2	12	0.91	19	1.44
5	8.05	16.0	48	3.00	32	2.00
6	8.05	14.5	23	1.59	32	2.20

Effect of variation in pH. Glucose uptake and lactic acid formation were studied at different pH's (Table 14). A displacement of the pH to 6.4 reduces the glucose uptake, but affects the lactic acid formation to a lesser extent; a smaller displacement in

the alkaline direction does not seem to affect the activity essentially.

Effect of variation in glucose concentration. Without glucose in the incubation fluid, only small quantities of lactic acid are formed, which in this case originates from glycogen or phosphate esters present in the tissue (Table 15). More lactic acid is formed with very low concentrations, but a maximum is reached at 50 mg %. Even concentrations as high as 500 mg % produced no further increase.

Table 15.

Effect of varied glucose concentration on lactic acid formation in slices of epiphyseal cartilage. Standard conditions, pH 7.4; temperature 37° C; incubation time 60 minutes.

Series no.	Glucose concentr.	Total amount of glucose in reaction mixture	Weight of slices	Lactic acid formed	
	mg %	µg	mg	µg	mg/g
			13.7	6.0	0.44
I	0	0	11.4	5.0	0.44
			14.4	3.7	0.26
			14.0	8.1	0.58
II	12.5	25	18.0	14.2	0.79
			12.3	15.0	1.22
			12.6	15.2	1.21
III	25.0	50	15.2	19.1	1.26
			14.8	19.7	1.33
			11.8	23.9	2.03
IV	50.0	100	14.6	31.2	2.14
			11.6	18.9	1.63
	150.0	300 mean values from table 11.			2.02
			16.8	36.4	2.16
V	500	1000	17.9	34.2	1.90
			16.7	39.3	2.36

Different hexoses as substrate. The amount of lactic acid formed in the presence of galactose is comparatively small. Fructose gives variable results; in one experiment the same amount as with galactose, in another considerably more (Table 16). Mannose and glucose produce approximately the same amount of lactic acid; glucose-1-phosphate sligthly less. With glucose as substrate nei-

Table 16.

Lactic acid formation with different hexoses as substrate in slices of epiphyseal cartilage. Standard conditions, hexose concentration 150 mg %; pH 7.4; temperature 37° C; incubation time 60 minutes.

Series no.	Substrate	Weight of slices	Lactic acid formed	
		mg	μg	mg/g
I	Galactose	14.0	11.2	0.80
		14.0	12.2	0.87
II	Fructose	11.4	16.2	1.42
		16.5	13.2	0.80
III	Mannose	16.3	35.3	2.16
		15.7	35.2	2.28
		15.0	29.4	1.96
IV	Glycogen	18.2	11.7	0.64
		23.0	9.2	0.38
V	Glucose-1-phosphate	19.7	35.3	1.80
		18.5	27.4	1.48
		17.4	34.1	1.96
VI	Glucose + ATP	14.4	33.2	2.30
		17.8	43.0	2.42

Table 17.

Effect of enzyme inhibitors on glucose uptake and lactic acid formation in slices of epiphyseal cartilage. Standard conditions, glucose concentration 150 mg %; pH 7.4; temperature 37° C; incubation time 60 minutes.

Series no.	Rat no.	Inhibitor added	Weight of slices	Glucose used		Lactic acid formed	
			mg	μg	mg/g	μg	mg/g
I	1	NaF 0.005 M	17.2	37	2.15	27.3	1.59
	2		19.0	22	1.16	29.5	1.55
	3		19.2	56	2.92	31.5	1.63
II	4	NaF 0.02 M	14.6	14	0.96	10.4	0.71
	5		14.0	0	0	11.0	0.78
	6		9.9	14	1.40	7.6	0.77
III	7	KCN 0.001 M	16.3	31	1.90	29.0	1.78
	8		19.5	33	1.70	42.3	2.18
	9		17.4	35	2.02	51.1	2.94
IV	10	CH_2JCOOH 0.001 M	16.4	0	—	0	—
	11		13.9	0	—	0	—

ther glycogen nor ATP (0.004 M) had any noticeable effect on the lactic acid formation.

Effect of inhibitors. The results of adding the well known selective inhibitors of glycolysis are presented in Table 17.

Sodium fluoride in a final concentration of 0.005 M was only slightly inhibitive; fluoride in a final concentration of 0.02 M had a considerably stronger inhibitory effect; iodo-acetic acid in a final concentration of 0.001 M blocked the lactic acid formation completely. Cyanide had no ostensible effect.

Effect of insulin. In one experiment 0.1 unit insulin was added to the incubating fluid. No effect on the glucose uptake or the lactic acid formation could be noted. In the following experiment four rats, 1 hour before being killed, each received 20 units insulin subcutaneously. The epiphyseal plate was sliced in the usual manner and the slices incubated for 1 hour in the standard medium. It is obvious from Table 18 that the glucose uptake increased considerably, while the lactic acid formation remained unchanged.

Table 18.

Glucose uptake and lactic acid formation in slices of epiphyseal cartilage from rats, each of which had received 20 units of Insulin one hour before they were killed. Standard conditions, glucose concentration 150 mg %; pH 7.4; temperature 37° C, incubation time 60 minutes.

Rat no.	Weight of slices	Glucose used		Lactic acid formed	
		µg	mg/g	µg	mg/g
1	16.6	88	5.30	30.3	1.88
2	9.7	48	4.95	16.8	1.73
3	12.8	52	4.06	26.2	2.05
4	12.2	54	4.44	15.2	1.24

The epiphyseal plate from rats, pretreated in a similar manner with 20 units of insulin, was incubated for different periods and the glucose uptake noted. Identical controls were run on slices from rats not injected with insulin. The glucose uptake in slices from insulin-treated animals was considerably higher than from control animals, the effect being especially pronounced during the first 30 minutes of incubation (Fig. 4).

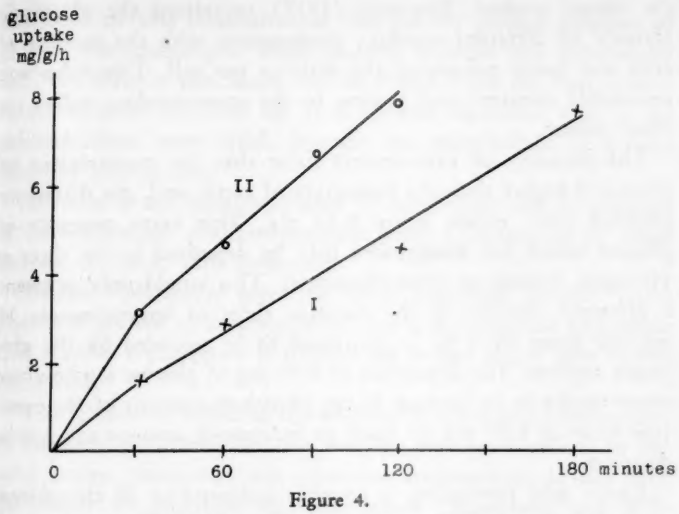

Figure 4.

Glucose uptake by slices of epiphyseal cartilage. Effect of insulin. Standard reaction mixture. Slices from 21 day old rats. Curve I: controls. Curve II: insulin injected rats.

C. *Discussion.*

The glycolytic activity of epiphyseal cartilage is estimated by chemical analysis of the amount of lactic acid formed in slices incubated in a salt solution. All values for the glycolytic activity are expressed as mg lactic acid formed per gram tissue per hour. When comparisons are to be made, the conventional Q-value — acid formation expressed as cmm CO_2 per mg dry weight per hour — is more useful. The cartilage contains 80 % water, consequently 2 mg lactic acid per gram wet weight corresponds to 0.01 mg per mg dry weight. 1 μM lactic acid (equal to 1 μEqv or 90 μg lactic acid) is equivalent to 22.4 cmm CO_2, thus giving a Q-value of 2.49 cmm CO_2 for epiphyseal cartilage. Approximately the same value was found by Bywaters (1937) for foetal rib and knee from cat and rabbit. Dickens and Weil-Malherbe (1936) found 1.85 for articular cartilage from rat; Lutvak-Mann (1940) 0.65 for cartilage from calf. This variation can presumably be ascribed to differences in the number of cells in

the tissues studied. Bywaters (1937) correlated the glycolytic activity of different cartilage preparations with the number of cells and hence calculated the activity per cell. This value was reasonably constant and is close to the corresponding values for other tissues.

The majority of experiments show that the consumption of glucose is higher than the formation of lactic acid, the difference between their means being 0.53 mg. This extra quantity of glucose which has disappeared may be deposited in the slices as glycogen, hexose- or triosephosphates. This would only augment a glycogen content in the cartilage slices of approximately 10 mg per gram by 5 % — too small to be recorded by the glycogen analysis. The deposition of 0.53 mg of glucose as phosphate esters results in an increase in the phosphate content of the cartilage slices of 0.09 mg/g. Such an increase in amount can not be detected.

Lactic acid formation, is roughly independent of the glucose concentration of the incubation medium. No difference between the lactic acid formation with 50 and 500 mg % glucose was noted. This fact suggests that the utilization of the glucose is not determined by its rate of difussion into the slices.

The first stage in the cellular utilization of glucose is via the hexokinase reaction:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP}$$

where hexokinase catalyzes the transport of phosphate to glucose in the 6-position. Hexokinase has a very great affinity for glucose — the Michaelis-Menten constant is 5×10^{-5} M glucose for brain hexokinase (Weil-Malherbe and Bone 1951). This corresponds to 0.9 mg % and even the lowest concentration used for the incubation of the cartilage slices (Table 15) should be sufficient to catalyse this reaction at a maximal rate.

Hexokinase catalyzes the phosphorylation of a limited number of hexoses. In accordance with this fact it has been found that cartilage forms an equal amount of lactic acid with glucose or mannose as substrate. Fructose forms less lactic acid than glucose. Meyerhof and Wilson (1948) found using brain extracts that glucose and fructose of initial concentration 2×10^{-2} M (360 mg %), were phosphorylated at approximately the same rate. With

one tenth of this concentration, the rate of phosphorylation of glucose was unchanged, while that of fructose was reduced by half. No definite conclusion can be drawn from the two experiments reported in Table 16. It is obvious, however, that as the concentrations were equal, fructose was metabolized at a considerably slower rate.

Galactose is not phosphorylated by hexokinase and its presence in the incubation medium produces only a comparatively small increase in lactic acid formation.

Glucose-1-phosphate gives less lactic acid than glucose. Phosphatase is present in cartilage slices and it is reasonable to suppose that glucose-1-phosphate is hydrolyzed before it can give rise to lactic acid.

With glucose as substrate the addition of adenosine triphosphate to the incubation medium was without noticeable effect. In general hexose phosphates and adenosine triphosphate do not have the ability to penetrate cell membranes (Rosenberg and Wilbrandt 1952, Boyland 1938) and these substances can not therefore be expected to take part in or activate intracellular processes.

Only small quantities of lactic acid are formed if hexose is absent as a substrate in the incubation medium. In these cases the main source of lactic acid is provided by the intracellular glycogen. The addition of glycogen to the incubation medium did not enhance the lactic acid formation which might be expected considering the fact that glycogen, having a molecular weight of 200—500.000, would have to penetrate the cell membrane. Epiphyseal cartilage contains about 10 mg glycogen per gram, sufficient to form a much larger quantity of lactic acid than that found after 1 hour's incubation. The rate of glycogen decomposition is ⅓ to ⅕ that of glucose. It remains to be established why glycogen is metabolized so much slower by the first two enzymes of phosphorlytic glycogen break down than by the other glycolytic enzymes. The problem has been investigated with the aid of homogenates of epiphyseal cartilages and is discussed in Chapter VII and VIII.

The experiments with glucose in the incubating fluid revealed that the quantity of glucose consumed corresponds fairly well with the quantity of lactic acid formed — as previously mentioned slightly more glucose disappears. This fact suggests that

glucose is essentially the source of lactic acid. Had the glycogen also been metabolized, the quantity of lactic acid formed would have been larger than the quantity of glucose consumed.

Cartilage tissue has certain metabolic characteristics. The lactic acid formation *in vitro* is independent of anaerobic and aerobic conditions and the oxygen consumption is low. This type of metabolism recalls that found in tumour and embryonic tissues (Warburg 1926) and in kidney marrow (Dickens and Weil-Malherbe 1936).

It will serve a useful purpose to discuss the type of metabolism involved in epiphyseal cartilage on a histological basis. Cartilage is avascular and is dependent of diffusion from the surrounding blood, supplying the tissue with the nutrients required. A reasonable assumption would appear to be that prior to the formation of a bone centre in the epiphyseal part of the bone, diffusion into the epiphyseal disc probably proceeds from the adjacent vascularized metaphysis. When this bone centre has developed, diffusion can also proceed into the opposite surface. Histologically it is seen, however, that this surface is covered at an early stage by a layer of bone which may form a barrier to diffusion. Considering the conditions prevailing before this bone centre has developed it can be assumed that diffusion can only proceed from the diaphyseal surface. The maximal thickness of the cartilage compatable with nutritional requirements is dependent on the metabolic rate, the rate of diffusion of the substances into the tissue and the influence of the concentration on the metabolism and other factors. This calculation, which would be very complicated for many substances, has been made for glucose for which the concentration and consumption are known. According to Hill (1929), the maximum thickness of a tissue nourished by diffusion from one side only is expressed by the formula:

$$1) \quad b = \sqrt{\frac{2 \cdot k \cdot y_0}{a}}$$

where k is the diffusion constant, y_0 the constant surface concentration of glucose and a the glucose consumption of the cartilage per unit of time. When 0.52×10^{-5} cm^2 sec^{-1} (Intern. Critical Tables 1929) is substituted for k, 0.1 % for y_0 and 2 mg

per g wet weight per hour for a, and all values are converted to g, cm, and minutes then

$$2) \quad b = \sqrt{\frac{2 \cdot 3{,}1 \cdot 10^{-4} \cdot 10^{-3}}{3{,}3 \cdot 10^{-5}}} = 1{,}4 \text{ mm}$$

The thickness of the epiphyseal plate in histological preparations measured approximately 1 mm. There is a low concentration of glucose in the intercellular fluid in those areas of the cartilage farthest from the diaphyseal surface, i. e. in the cell division zone and the beginning of the proliferation zone. The experiments have shown, however, that the lactic acid formation in cartilage is, roughly speaking, independent of the glucose concentration. The cell requirement can therefore be satisfied by low sugar concentrations.

The energy requirement of the epiphyseal cartilage can be essentially satisfied by glycosis. The energy liberated in the decomposition of glucose to lactic acid can be calculated to be 36.000 cal. per mol. glucose. One mg glucose corresponds to 0.2 calories. The epiphyseal cartilage consumes about 2 mg glucose per gram per hour, corresponding to 0.4 cal. per gram per hour. As mentioned before, the oxidative metabolism is low with a Q_{O_2} of about 0.005 μl, which corresponds to approximately 1 μl per gram wet weight per hour (Bywaters 1937, Lutvak-Mann 1940, Hills 1940). Six mol. oxygen are consumed and 672.000 cal. liberated during the oxidation of 1 mole glucose to carbon dioxide and water. For each μl oxygen consumed, 5×10^{-5} cal. are liberated; consequently the oxidative metabolism in cartilage accounts for only 1 % of the energy requirement. An equal contribution of energy from the oxydative metabolism and the glycogen presupposes an oxygen consumption of $\frac{0.4}{5 \cdot 10^{-3}} = 80$ μl per gram per hour.

The energy liberated during the fermentation of glucose to lactic acid is converted almost quantitatively into phosphate bond energy (Lipmann 1941). The net gain for each glucose molecule metabolized is two energy rich phosphate bonds. The metabolic energy is passed by means of adenylic acid into pyrophosphate bonds where it can be utilized for different purposes. Part of this energy is used in the epiphyseal plate for the synthesis of glycogen.

The time spent by the cells in the epiphyseal cartilage from their development in the cell division zone to their disappearance in the calcifying zone has been determined. On this basis it is possible to estimate how much glycogen is synthetized in the cartilage and hence the amount of energy (available after glycolysis) which is required for glycogen synthesis.

It has been found that the glycogen content of the epiphyseal plate is 10 mg per gram wet weight. Histochemical investigations show that glycogen is unevenly distributed, the cells in the transition zone between the proliferation and hypertrophy zones containing more than the other cells. It is reasonable to suppose that the cells in the cell division zone originally have a relatively low glycogen content which increases as the cells mature until a maximum is reached in the transition to the hypertrophy zone. The glycogen content in this area, estimated at 20 mg/g, is therefore higher than in the rest of the cartilage. Previously it has been calculated that the cells take 20—30 hours to pass through the proliferation zone during which time the glycogen is synthetized. Epiphyseal cartilage forms 2 mg lactic acid per hour and will during the same time form enough energy rich phosphate bonds to phosphorylate twice this amount of glucose i. e. 4 mg. In the course of 20—30 hours there will be enough energy to form 80—120 mg glycogen. Only 20 mg, however, is formed, indicating that only approximately 20 % of the available energy is utilized for glycogen synthesis.

The effect of glycolytic inhibitors is consitent with their effect in other tissues. Cyanide had no effect on lactic acid formation. The fact that cyanide did not increase the lactic acid formation is in good agreement with results obtained in preliminary experiments where no difference between the lactic acid formed under aerobic and anaerobic conditions was found. The Pasteur effect (reduced glycolysis in an oxygen atmosphere) is not present in cartilage. Tumour and embryonic tissues exhibit similar characteristics (Warburg 1926), although not so pronounced.

Addition of fluoride, at a concentration of 0.005 M has no appreciable effect on the lactic acid formation under the experimental conditions. A comparison with the effect of fluoride on *in vitro* calcification may be of value in this respect. With inorganic phosphate in the medium and a fluoride concentration of

M/10.000, the calcification of cartilage slices is completely inhibited. At this low fluoride concentration, glycolysis in cartilage slices is hardly affected. Of course it is possible that the fluoride inhibition of *in vitro* calcification may have another mode of action, e. g. it may affect the organic ground substance or prevent crystal growth.

Chapter VII.

Lactic Acid Formation in Homogenates of Epiphyseal Cartilage.

Experiments in which slices from epiphyseal cartilage were incubated showed that the rate of lactic acid formation *in vitro* was considerably lower when the slice had to metabolize its own glycogen.

The following experiments were carried out to find the cause of the slow decomposition of glycogen. There are several possibilities, viz: the activity of phosphorylase is lower than that of the other glycolytic enzymes; the cells do not contain enough coenzymes for these reactions (adenylic acid and/or glucose-1.6-diphosphate); the concentration of inorganic phosphate in the cells is too low. In experiments with slices, permeability is a factor difficult to control and with unknown inherent possibilities for variation. These difficulties are avoided by using homogenates. Any substrate can be brought in direct contact with its specific enzymes, the necessary coenzymes can be added and the incubating system varied with respect to ionic composition, pH and the inhibitors whose effect should be studied.

A. *Material and Methods.*

The epiphyseal heads from the distal part of the tibiae and the proximal part of the femurs of young rats were dissected out and all soft parts removed. They were cut into thin slices, weighed and placed in mortars containing small quantities of isotonic KCl solution. The mortars were placed in melting ice.

Cartilage from 4—6 rats provided sufficient material. Isotonic KCl solution was added to give a 10 % homogenate (9 ml solution per g tissue). The slices were then pounded heavily with a pestle for 5—10 minutes until a relatively homogeneous tissue suspension was obtained. The suspension was subjected to brief centrifugation to make pipetting possible, and the desired volume of homogenate was measured from the turbid supernatant.

A few comments on this procedure are necessary. Work with epiphyseal plates was found to involve practical difficulties. The weight of tibial epiphyseal plates ranges from 10—15 mg. Fifteen to twenty rats would therefore be required in each experiment to provide a working amount of homogenate (400 mg tissue gives 3.6 ml homogenate). It was therefore decided to use the entire epiphyseal head from the proximal tibial and distal femoral ends. In 10—12 day old rats the epiphyses of both these bones are still cartilaginous; the cartilage cells in the central areas are hypertropic. Twelve days after birth the epiphyseal ossification centre begins to develop, but remains small during the next 4 days. Th tissue used consists mainly of cartilage cells and the morphological picture shows that a large number of the cells are typical of those in endochondral calcifying cartilage.

With such tissue as cartilage it is difficult to judge if the degree of homogenization is uniform in different experiments. Consequently the results show appreciable variation. Conclusions when a new variable was introduced were therefore only drawn when the activity deviated considerably from the mean values. A control experiment of the systems activity was run concurrently with the experiments in which the composition of the medium varied. The relative activities could thus be established.

Incubation system. Glycolysis was investigated in a medium with the following composition: 0.0024 M potassium phosphate buffer, pH 7.5, 0.025 M potassium bicarbonate, 0.04 M nicotinamide, 0.0033 M adenosine triphosphate, 0.0002 M diphosphopyridine nucleotide, 0.01 M fructose-1.6-diphosphate, 0.0067 M magnesium chloride, 0.01 M potassium fluoride, 0.005 M potassium pyruvate. This medium was evolved by LePage (1948) and gives optimal conditions for anaerobic glycolysis with homogenates of brain, kidney and liver tissues, and for homogenates of Flexner-Jobling tumour in rats. This system also offers optimal

conditions for *in vitro* anaerobic phosphorylation processes with brain and tumour tissues.

Incubation was carried out at 37° C in standard Warburg vessels with air in the gas phase. The total volume was always 3 ml and 0.3—0.5 ml of tissue homogenate was employed. The amount of homogenate was partly determined by the number of animals at disposal and partly by the size of the activity expected in the actual experiments. The reaction was stopped by the addition of 2 ml of 10 % trichloroacetic acid. The controls, in which trichloroacetic acid was added before the homogenate, were set up concurrently. After centrifugation, an aliquot of supernatant was taken for lactic acid analysis as prescribed by Barker and Summerson (1941).

*Reagents.**) Adenosine triphosphate, barium salt from Sigma, 95—100 % pure by phosphate analyses. From the barium salt the potassium salt was made as directed in «Biochemical Preparations» (1949). Diphosphopyridine nucleotide from Sigma, approximately 65 % pure (corrected for purity in the calculation of molarity). Fructose-1.6-diphosphate and fructose-6-phosphate, barium salts from Schwartz Labs. Purified according to Neuberg et al. (1943). Glucose-1-phosphate, potassium salt from Sigma. Glucose-6-phosphate, barium salt from Sigma. Pyruvic acid, sodium salt from Hoffman la Roche. Glycogen, pure from Hoffman la Roche and impure from British Drug House. The latter was purified according to Illingworth et al. (1952) and Somogyi (1934). All the other reagents were analytically pure.

B. *Results.*

Glucose and glycogen are the main substrates for *in vivo* glycolysis in the tissues. The glycolytic cycle consists of 12 consecutive steps and the activity is dependent on the activity of the same number of intermediate enzymes, several organic coenzymes, activators and specific ions. Therefore the rate of reaction of dis-

*) The following abbreviations are used: adenosine triphosphate — ATP, adenosine diphosphate — ADP, diphosphopyridine nucleotide — DPN, glucose-1 phosphate — G-1-P, glucose-6-phosphate — G-6-P, fructose-6-phosphate — F-6-P, fructose-1.6-diphosphate — F-1.6-DP, glucose-1.6-diphosphate — G-1.6-DP.

integrated tissue obviously varies according to the experimental conditions. In the present system several of the steps in the triose part of the cycle are disconnected by the addition of fluoride. Fluoride selectively inhibits enolase which catalyzes the conversion of 2-phosphoglyceric acid to phosphopyvuric acid. The glycolysis is therefore blocked at this step and excess pyruvic acid is added to enable measurement of the activity of the preceding steps. An equivalent amount of pyruvic acid is reduced to lactic acid for every mole of 1.3-diphosphoglyceric acid formed. Diphosphopyridine nucleotide serves as the hydrogen transporter. The reaction scheme is presented in Figure 5. The amount of lactic acid formed during incubation is therefore a measure of the activity of this short-circuited system.

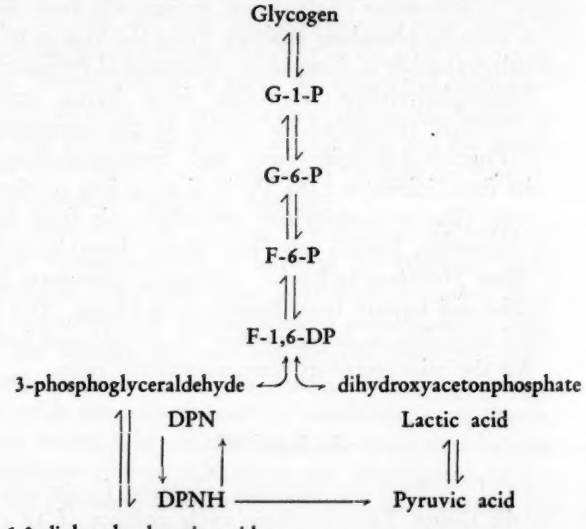

Figure 5.
Schematic reaction chain in the system for homogenates.

The conditions for optimal glycolytic activity have already been worked out for several tissues (Novikoff et al. 1948, LePage 1948, Stoesz and LePage 1949, Utter 1950) and in essence these have been used in the present investigations. No systematic in-

vestigations of the effect of variations in the composition of the medium on the activity of the system were made, but a few experiments were performed to determine whether the system reacted in a similar fashion to other tissues in this respect.

Variations in initial pH. The initial pH of the incubation medium was varied from 7.0 to 8.0 by adding small quantities of hydrochloric acid or sodium hydroxide prior to the addition of homogenate. The pH was checked with a glass electrode. The results presented in Table 19 show that the lactic acid formation did not undergo any important changes. In a number of experiments the pH was controlled after incubation by means of phenol red and no noticeable deviation from the initial pH was observed.

Table 19.

Effect of initial pH on lactic acid formation in homogenates of epiphyseal cartilage. Reaction mixture as described in text. Fructose-1.6-diphosphate as substrate; 0.4 ml homogenate; temperature 37° C; incubation time 60 minutes.

Experiment no.	pH	Net lactic acid formed
		µg
H-28	7.0	141
	7.4	156
	7.8	142
H-25	7.1	116
	7.5	118
	7.95	103

Effect of adenosine triphosphate (ATP). ATP is known as a coenzyme or carrier for enzymic phosphate transfer. In glycolysis ATP is required as a donor for the phosphorylation of glucose in the hexokinase reaction and F-6-P in the phosphofructokinase reaction. Adenosine diphosphate (ADP) is necessary as acceptor in the triosephosphokinase and in the phospho-enol transphosphorylation reactions. With F-1.6-DP as a substrate, ADP functions only as an acceptor. In tissue homogenates ATP liberates phosphate groups partly by transphosphorylation, and partly by hydrolysis of the phosphatases present. In some tissues this is due to a specific adenosine triphosphatase, while in cartilage there is an unspecific «alkaline» phosphatase. The same glycolytic ac-

tivity is usually obtained by the addition of ATP or ADP to tissue homogenates. The exclusion of ATP from the incubation fluid for cartilage homogenate results in a 40 % reduction of the lactic acid formation (Table 20). Similar results were obtained by Stoesz and LePage (1949) for liver homogenates.

Table 20.

Effect of ATP and DPN. Reaction mixture described in text. Fructose-1.6-diphosphate as substrate. 0.3 ml homogenate from 12 day old rats, incubation time 60 minutes. (Exp. no. H-20.)

Reaction mixture	Net lactic acid formed
	μg
Complete	136
— ATP	85
— DPN	33

Effect of diphosphopyridine nucleotide (DPN). This substance is required as a coenzyme for hydrogen transport in glycolysis. DPN acts as hydrogen acceptor in the triosephosphate dehydrogenase reaction where 3-phosphoglyceraldehyde is oxidized to 1.3-diphosphoglyceric acid. In the lactic acid dehydrogenase reaction DPNH acts as donor and the hydrogen is transferred to pyruvic acid which is reduced to lactic acid. When DPN is not added to the incubation medium, the lactic acid formation is reduced by 70 % (Table 20) — a fact in accordance with results on other tissues (LePage 1948 and Stoesz and LePage 1949).

The effect of nicotinamide was not studied separately. It is known from work with other tissues (Novikoff et al. 1948) that this substance protects DPN against enzymatic decomposition and hence increases the activity of homogenates.

Effect of pyruvate. In the absence of pyruvate the system is inactive. No lactic acid was formed in an experiment in which pyruvate was omitted.

Effect of varied incubation time. The results are presented in Figure 6. The lactic acid formation was measured in individual incubating vessels and the reaction was stopped (trichloroacetic acid addition) after 15, 30, 45 and 60 minutes incubation. The

lactic acid formation was found to be a linear function of time for the first thirty minutes. The cause of the subsequently slower rate was not studied more closely. Similar observations, however, were made with homogenates of other tissues. In the present work, where the aim was to compare the effect of variations in

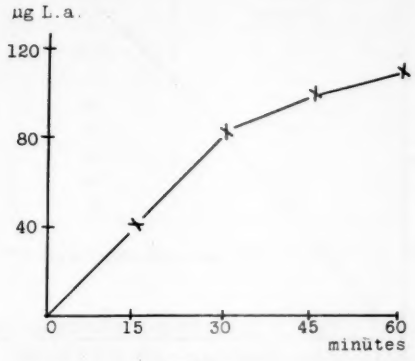

Figure 6.

The influence of the incubation time on the lactic acid formation. Reaction mixtures as described in the text. Fructose-1.6-disphosphate as substrate, 0.3 ml homogenate, pH 7.5, temperature 37° C. (Exp. no. H-58.)

the composition of the incubation medium, an incubation time of 60 minutes was used to allow maximal lactic acid production. The activity was measured at different intervals in a number of experiments where it might decrease at an even faster rate during incubation.

Effect of the amount of homogenate. Satisfactory agreement was obtained between the amount of tissue in the incubating vessels and the amount of lactic acid formed (Fig. 7). More lactic acid was formed during incubation in this series of experiments due to the entire epiphyseal head from 27 day old animals being used to obtain an adequate amount of homogenate. The epiphyseal ossification centre in the epiphyseal head of the animals had reached a considerable size at this age. The homogenate will therefore contain some red bone marrow which causes the larger formation of lactic acid. The red marrow is rich in cells with properties other than those in cartilage cells. The experiment further

illustrates that the method responds well to the amount of tissue and the necessity in this type of experiment of defining the type of tissue.

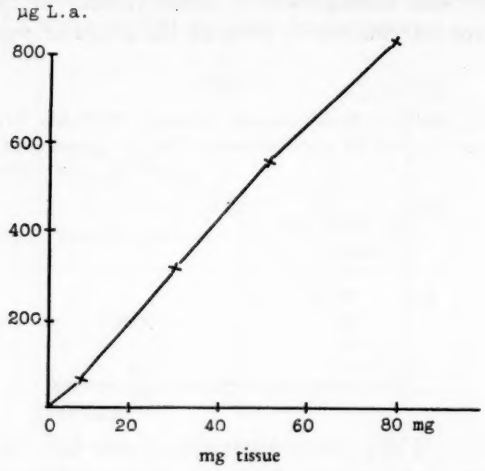

Figure 7.

Effect of varying amounts of tissue on lactic acid formation. Reaction mixture as described in the text. Fructose-1.6-diphosphate as substrate, pH 7.5, temperature 37° C, incubation time 60 minutes. The homogenate was prepared from 27 days old rats. (Exp. no. H-53.)

Age of animals. It was important to determine on the basis of the previous experiments to what extent the developmental stage affects the activity. Homogenates of the entire epiphyseal head from the proximal tibial and the distal femoral ends of animals of different ages were prepared and incubated in the standard manner. The results are presented in Table 21. All the homogenates were incubated for 30 minutes during which time the activity was maintained. The lactic acid production undoubtedly increased with age. The very low activity after 7 days may to some extent be due to variations inherent in this type of study and to the fact that the main part of the epiphyseal head of animals at this early age consists of small-celled hyaline cartilage which contains fewer cells than "calcifying cartilage" ("growth cartilage" is possibly a better term). The lactic acid increased during the following 2

Table 21.

Effect of age on lactic acid formation. Reaction mixture described in text. Fructose-1.6-diphosphate as substrate, 0.3 ml homogenate incubated for 60 minutes. (Exp. no. H-59 and H-60.)

Age of rats	Net lactic acid formed*)
days	μg
7	33
10	79
11	105
13	97
15	124
21	224

*) Each value represents the mean of two animals.

days, remained relatively constant at a higher level during the 5 day period, 10th—15th day, and increased still more during the following 12 days.

Ten to fifteen day old rats were used in the majority of the experiments, but older rats were occasionally used. When the results had any important bearing for the ensuing discussion, identical experiments were performed with homogenates from younger animals.

Effect of substrate. F-1.6-DP was exclusively used as the substrate in all the preliminary experiments that have been described above. The system therefore functions in its simplest form (cf. Fig. 5), the four glycolytic enzymes functioning being aldolase, triosephophate dehydrogenase, triosephosphate isomerase and lactic acid dehydrogenase. The reaction cycle can be systematically extended by substituting other hexose phosphates in the glycolytic cycle for F-1.6-DP. With F-6-P present, phosphofructokinase catalyzes transphosphorylation from ATP to the 1-position in F-6-P; with G-6-P present the enzyme hexose isomerase is required to catalyze the transformation to F-6-P; with G-1-P present phosphoglucomutase is additionally required to catalyze the conversion of 1-phosphate to 6-phosphate.

The results of the incubation experiments with different hexose phosphates are presented in Table 22. In all the experiments the

Table 22.

Lactic acid formation in homogenates of epiphyseal cartilage with different hexose phosphates as substrates. Results from experiments in which the same homogenate was incubated with two different hexose phosphates.

Experiment no.	Age of rats	Amount of homogenate	Net lactic acid formed			
			F-1.6-DP	F-6-P	G-6-P	G-1-P
	days	ml	µg	µg	µg	µg
H-23	14	0.4	209	111	—	—
			284	110		
H-35	17	0.4	443	148	—	—
			443	148		12
H-32	14	0.4	246	—	—	14
H-36	17	0.4	394	—	—	21
			384			
H-54	17	0.4	—	98	97	—
H-75	13	0.5	—	61	106	—

largest amount of lactic acid was formed with F-1.6-DP as the substrate. Only ⅓ of this amount was formed when either F-6-P or G-6-P was the substrate and even less with G-1-P.

One control experiment was performed in which muscle homogenate from adult rats was used and where G-1-P was the substrate. It is well known that in muscle tissue glycogen is rapidly broken down to lactic acid under anaerobic conditions. If none of the enzymic reactions catalyzing the conversion of G-1-P to F-1.6-DP is rate limiting in the muscle, the lactic acid formation will presumably be equally large with G-1-P or F-6-P as substrate. This assumption was confirmed (Table 23). Addition of a small amount of F-1.6-DP did not increase the lactic acid formation when G-1-P was the substrate.

According to these results, phosphoglucomutase has apparently less activity than the other glycolytic enzymes in the cartilage. Najjar (1948) has shown, however, that crystalline phosphoglucomutase requires cysteine for its activity and that the activity is inhibited by fluoride ions. Mutase is active in crude muscle ex-

Table 23.

Lactic acid formation in muscle homogenate. Standard reaction mixture with different hexose phosphates as substrate. 0.3 ml homogenate of muscle from old rat. Incubated at 37° C for 30 minutes.

Substrate	Substrate concentration in reaction mixture	Net lactic acid formed
	M	μg
F-6-P	7×10^{-3}	938
G-1-P	7×10^{-3}	1060
F-1.6-DP+ G-1-P	2×10^{-4} 7×10^{-3}	1130
F-1.6-DP	2×10^{-4}	103

tracts without the addition of cysteine. This does not necessarily apply to cartilage homogenates and it was demonstrated in later experiments that when fluoride ions were excluded and cysteine was added to homogenates of epiphyseal cartilage, then with G-1-P as substrate the lactic acid formation was of the same size as with other hexose monophosphates (Table 24).

Table 24.

Lactic acid formation in epiphyseal homogenate with G-1-P as substrate. Effect of fluoride and cysteine. Standard reaction mixture. 0.5 ml homogenate. Incubated at 37° C for 30 minutes.

Experiment no.	Age of rats	Additions or omissions in reaction mixture	Net lactic acid formed
	days		μg
H-63	17	+ 0.03 M cysteine	12
H-64	19	+ 0.03 M cysteine	
		— KF	73
H-81	10	+ 0.03 M cysteine	
		— KF	83

C. *Discussion.*

In homogenates the amount of lactic acid formed with F-1.6-DP as substrate is considerably higher than that found for slices of epiphyseal cartilage. When converted to mg of lactic acid formed per gram wet weight of cartilage per hour, the results obtained with homogenate range from 4—10 mg, with slices from 2—3 mg. With F-6-P or G-6-P, the lactic acid formation ranges from 2.5 to 4 mg per gram per hour which correlates better with the activity of the slices. With F-1.6-DP aldolase is saturated with its substrate, whereas the substrate is formed in low concentrations when monophosphates are added. Another possibility is that phosphofructokinase is the rate limiting step. The cause of the lower activity with the monophosphates may be attributed to either or both of these factors.

Equal quantities of lactic acid were formed with G-6-P and F-6-P. Hexose isomerase is therefore not rate limiting under these experimental conditions.

The results for G-1-P were consistent with the effects of fluoride and cysteine on phosphoglucomutase. With this enzyme under optimal conditions, the system showed just as high an activity as with the other two hexose monophosphates tested. Considering the low lactic acid formation in slices of epiphyseal cartilage which were forced to metabolize their own glycogen, or in homogenates with glycogen as substrate (see chapter VIII), these results suggest that the phosphorylase system is the rate limiting step in the breakdown of glycogen. In experiments with homogenates and glycogen as substrate, G-1-P is formed by the phosphorylase reaction at a slow rate and a low substrate concentration for the phosphoglucomutase would therefore be expected. In this situation the low lactic acid formation might be explained by a combination of a low phosphorylase and phosphoglucomutase activity, the latter mainly due to low substrate concentration. Thus the system is not showing the phosphorylase activity alone.

These possibilities were subjected to closer investigation and will be discussed in the following chapter.

Chapter VIII.

Lactic Acid Formation in Homogenates of Epiphyseal Cartilage after Addition of Purified Enzyme Preparations.

So far no single enzyme in the glycolytic cycle has been found that can provide a reasonable explanation for the slow breakdown of glycogen in slices of epiphyseal cartilage. By measuring the lactic acid formation in homogenates after the addition of excess of phosphoglucomutase, or phosphorylase it should be possible to differentiate, which of these enzymes are rate limiting.

During work with phosphoglucomutase it was observed that the preparation used, probably contained other enzymes of the glycolytic system. Of these, aldolase and triosephosphate dehydrogenase were prepared and tested separately.

A. *Methods.*

The previously described incubation system was used (see page 44).

1) *Phosphoglucomutase.* This enzyme was prepared from rabbit muscle according to Colowick and Sutherland (1942). The final clear solution was tested for activity by Najjars method (1948). In this incubation system, 0.1 ml enzyme (dilution, 1 : 20) transformed G-1-P to the equilibrium mixture of 1- and 6-esters in 10 minutes (Fig. 8). The stock solution was stored in the refrigerator and kept its activity for several weeks.

2) *Phosphorylase* was prepared in crystalline form from rabbit muscle according to Green and Cori (1943) and Illingworth and Cori (1953) and then recrystallized twice. The crystals were kept in suspension in a cysteine-glycerophosphate solution (pH 6.7) at 4° C. The activity was tested according to Illingworth and Cori (1953) and the results are given in Table 25.

3) *Aldolase and triosephosphate dehydrogenase* were prepared as crystals from the same extract of rabbit muscle; aldolase according to Taylor et al. (1948), triosephosphate dehydrogenase according to Cori et al. (1948).

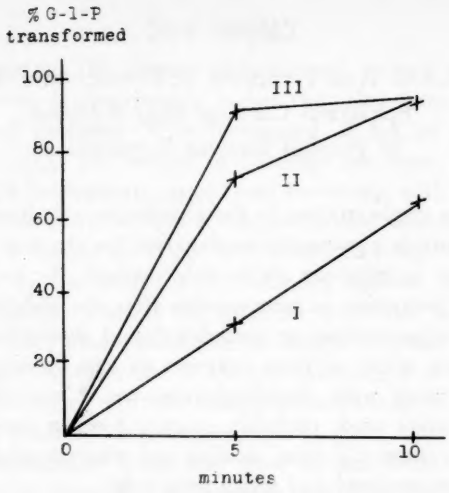

Figure 8.

Activity of phosphoglucomutase preparation. The reaction mixture had the following composition: glucose-1-phosphate 5×10^{-3} M $MgSO_4$ 1.5×10^{-3} M cysteine 0.025 M. 0.1 ml enzyme solution in a total volume of 0.4 ml. I: enzyme dilution 1 : 40, II: enzyme dilution 1 : 20, III: enzyme dilution 1 : 10.

Table 25.

Activity of phosphorylase. The reaction mixture had the following composition: 0.015 M cysteine, 0.02 M glycerophosphate, 1 % glycogen, 0.016 M glucose1-1phosphate, and 0.001 M adenylic acid, pH 6.8 in a total volume of 0.8 ml. 0.4 ml diluted enzyme added. After exactly 5, 10 and 15 minutes, 0.2 ml aliqouts were taken out for phosphorus determination by the Fiske-Subbarow method.

Time min.	per cent P liberated
5	10.9
10	18.1
15	24.8

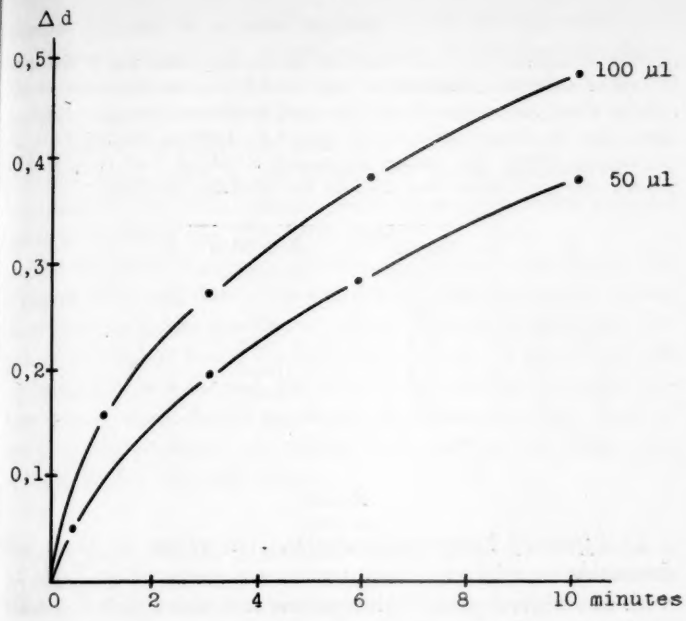

Figure 9.

Activity of triose phosphate dehydrogenase. The reaction mixture had the following composition: 0.3 ml 0.1 N sodium pyrophosphate pH 7.1; 0.1 ml = 0.6 mg DPN; 0.1 ml sodium fructose-1.6-diphosphate (6 mg P/ml); 0.05 ml aldolase suspension (diluted 1 : 20); 0.05 and 0.10 ml triose phosphate dehydrogenase (crystal suspension diluted 1 : 50); distilled water to a volume of 2.9 ml. At time 0 added 0.1 ml 5.4 % $Na_2HAsO_4 \cdot 7H_2O$ in water. Optical density read in the Beckman at 340 mµ. Room temperature (20° C).

Aldolase crystallized as needles and was stored in half saturated ammonium sulphate solution of pH 7.5 in the refrigerator. The activity was tested by the method of Sibley and Lehninger (1949) and the results are given in Table 26.

Triosephosphate dehydrogenase was prepared from the muscle extract after precipitation of the aldolase.

Crystals of the enzyme were prepared and stored in 0.66 saturated ammonium sulphate (pH 8.0) in the refrigerator. The activity was measured spectrophotometrically in the Beckman spectrophotometer according to Warburg and Christian (1943) (Fig. 9).

Table 26.

Activity of aldolase. 0.025 ml crystalline aldolase suspension was diluted with 150 ml of water. Given amounts of this solution were incubated for 30 minutes in a reaction mixture of the following composition: fructose-1.6-diphosphate 0.005 M, borate buffer 0.04 M (pH 8.6), hydrazin sulphate 0.056 M. Temperature 37° C. The reaction was stopped by adding 2 ml 10 % trichloroacetic acid and aliquots were analyzed for alkalilabile phosphate.

Enzyme dilution	Alkali labile phosphate
ml	μg
0.1	4.85
0.2	10.10
0.3	14.4

Results.

1) *Effect of Phosphoglucomutase.* The values for lactic acid formation in epiphyseal homogenates are presented in Table 27, with and without phosphoglucomutase and with F-6-P, F-1.6-DP or G-1-P as substrate. Without mutase, the values were consistent with those previously obtained; with mutase the lactic acid for-

Table 27.

Effect of phosphoglucomutase on lactic acid formation in homogenates of epiphyseal cartilage. Standard reaction mixture. 0.4 ml homogenate from 16 day old rats. Incubated at 37° C for 60 minutes.

Experiment no.	Substrate	Mutase added	Net lactic acid formed
		ml	μg
H-10	F-6-P	0	130
	F-6-P	0.1	456
	G-1-P	0	45
	G-1-P	0.1	681
H-37	F-1.6-DP	0	389
	F-1.6-DP	0.1	663
	G-1-P	0	14
	G-1-P	0.1	634

mation increased in all cases and the amount formed with G-1-P as substrate was as large as with the other two hexose phosphates. Two facts are evident from this experiment, namely: G-1-P is rapidly converted to G-6-P as expected and more lactic acid is thus formed. Secondly the lactic acid production with the other two phosphate esters is also augmented by the addition of mutase, showing that the phosphoglucomutase preparation contains other enzymes of the glycolytic cycle.

The results in Table 28 confirmed previous experiments (see Chapter VII) and showed in addition that the lactic acid formation increases as the amount of mutase becomes progressively larger, a maximum being reached with 150 µl. With 50 µl, 169 µg lactic acid was formed, the value to be expected from the mutase action alone. Small quantities of other glycolytic enzymes are probably present in the system, hence more of the preparation is required for maximal effect.

Table 28.

Effect of varying amount of phosphoglucomutase on lactic acid formation in homogenates of epiphyseal cartilage. Standard reaction mixture with G-1-P as substrate. 0.4 ml homogenate. Incubated at 37° C for 60 minutes. (Exp. no. H-11.)

Mutase added	Net lactic acid formed
ml	µg
0	18
0.025	75
0.050	169
0.1	351
0.15	573
0.2	493

This contamination of the mutase preparation was regarded as an advantage when the primary aim was to study the initial steps in the phosphorolytic decomposition of glycogen. The preparation did not contain phosphorylase — a finding in accordance with that of Colowick and Sutherland (1942). Addition of excess mutase ought to ensure the rapid conversion of G-1-P as soon as it is formed from glycogen when the amount of lactic acid formed should become an indication of the phosphorylase activity.

Next the lactic acid formation (resulting from addition of the mutase) from G-1-P and glycogen was compared. With G-1-P as substrate, the lactic acid formation increased to the same extent as in previous experiments; with glycogen as substrate the lactic acid formation remained small (Table 29). Under the present experimental conditions the phosphorylase system was therefore the rate limiting factor.

Phosphorylase catalyzes the equilibrium reaction:

$$\text{glycogen} + H_3PO_4 \overset{\text{phosphorylase}}{\rightleftharpoons} \text{G-1-P}$$

and an increase in the concentration of inorganic phosphate displaces the equilibrium to the right. The effect obtained by varying the concentration of inorganic phosphate is given in Table 30. The highest phosphate concentrations produced the largest lactic acid formation.

The influence of the incubation time on the lactic acid formation with glycogen as substrate was then investigated (Table 31). The rate in the first 30 minutes, although low as previously observed, is noticeably larger than in the succeeding 30 minutes.

In a number of experiments the composition of the incubation fluid was varied so as to find a system offering maximal phosphorylase activity.

Reducing substances (glutathione, cysteine, KCN) have been found to increase the activity of phosphorylase (Green and Cori 1943). In tissue and in tissue extracts phosphorylase can have two forms: phosphorylase a and b. The latter form is activated by adding adenylic acid (Cori and Cori 1940). In tissue extracts, phosphorylase a is converted to the b form by PR-enzyme (phosphorylase rupturing enzyme, Keller and Cori 1953). This transformation may possibly take place in cartilage homogenates.

There is also the possibility that mutase is inactive because of the absence of the coenzyme, glucose-1.6-diphosphate (LeLoir et al. 1948, Cardini et al. 1949, Sutherland et al. 1949). Standard preparations of G-1-P contain G-1.6-DP as an impurity in amounts sufficient to give maximal activation of the mutase, but too low to be detected by standard chemical methods (Sutherland et al. 1949). The G-1-P preparation used in the present experiments contained this coenzyme in amounts sufficient

Table 29.

Effect of phosphoglucomutase with glucose-1-phosphate and with glycogen as substrate. Standard reaction mixture. 0.4 ml homogenate from 15 day old rats. To each vessel was added 0.1 ml phosphoglucomutase (diluted 1 : 20). Incubation time 60 minutes. (Exp. no. H-13.)

Substrate	Substrate concentration	Net lactic acid formed
		μg
G-1-P	0.01 M	674
—»—	—»—	684
—»—	—»—	689
Glycogen	0.5 %	20
—»—	—»—	25
—»—	—»—	30

Table 30.

Effect of inorganic phosphate concentration on lactic acid formation with glycogen as substrate. Standard reaction mixture. Glycogen concentration 0.5 per cent. 0.4 ml homogenate incubated for 60 minutes.

Experiment no.	Ages of rats	Net lactic acid formed	
		Inorg. P 0.0024 M	Inorg. P 0.020 M
	days	μg	μg
H-49	12	0	20
H-50	17	5	24
H-51	17	13	44

Table 31.

Effect of incubation time on lactic acid formation in homogenates of epiphyseal cartilage with glycogen as substrate. Standard reaction mixture. Glycogen concentration 0.5 %. 0.4 ml homogenate incubated at 37° C.

Experiment no.	Age of rats	Net lactic acid formed	
		30 min.	60 min.
	days	μg	μg
H-44	15	10	16
H-46	14	14	19

to activate the phosphoglucomutase (Fig. 8). The amount of G-1.6-DP formed by decomposition of glycogen during the phosphorylation reaction, however, cannot be expected to be sufficient to activate the mutase added.

As a consequence of these considerations, cartilage homogenates were incubated in a medium with the following composition: 0.024 M potassium phosphate buffer, pH 7.5, 0.0025 M potassium bicarbonate, 0.04 M nicotinamide, 0.0033 M adenosine triphosphate, 0.0002 M diphosphopyridine nucleotide, 0.03 M cysteine, 0.0003 M adenylic acid, 0.0067 M magnesium chloride, 0.005 M potassium pyruvate. In a number of experiments glucose-1.6-diphosphate was also added to give a final concentration of 3×10^{-5} M. Glycogen at a concentration of 0.5 % served as substrate, and hexose phosphates at a concentration of 0.01 M were used as previously.

Table 32.

Lactic acid formation in homogenates of epiphyseal cartilage with glycogen as substrate. Composition of reaction mixture as described in text. 0.5 ml homogenate incubated at 37° C for 60 minutes.

Experiment no.	Age of rats	Net lactic acid formed
	days	μg
H-66	15	26
H-67	16	12
H-68	14	19
H-79	13	13
H-80	12	36
H-81	10	24
H-82	14	20
H-83	12	14

The results of experiments with glycogen as substrate are shown in Table 32. In some of the experiments the lactic acid formation was somewhat larger with this system than with that previously employed. The variation was probably due to differences in the degree of homogenization. Phosphoglucomutase had no effect on the lactic acid formation in this system. Excess mutase was added in experiments 81 and 82 (Table 32) and the lactic acid formation was 24 and 20 μg respectively. Mutase in the absence of cartilage

homogenate gave no lactic acid. As previously mentioned, higher activity is also obtained when G-1-P is a substrate for this system. Phosphoglucomutase augments lactic acid formation with G-1-P as substrate (se Table 28).

2) *Effect of Phosphorylase.* The results hitherto obtained indicate that phosphorylase might be the rate limiting enzyme in the breakdown of glycogen. To provide further evidence regarding this assumption, crystalline phosphorylase was prepared from rabbit muscle as described and the effect of the addition of small amounts of this enzyme on the lactic acid formation of cartilage homogenate with glycogen as substrate, was studied.

Table 33.

Effect of phosphorylase. Reaction mixture as described on p. 62. Glycogen as substrate (1 %); pH 7.5; temperature 37°; incubated for 30 minutes. 0.5 ml homogenate from 13 day old rats. Phosphorylase suspension diluted 1 : 50. (Exp. no. H-89.)

Substrate	Phosphorylase added	Net lactic acid formed
	µl	µg
Glycogen	0	29
—»—	50	65
—»—	200	73
G-1-P	0	82

The experimental results are given in Table 33. Without the addition of phosphorylase the net lactic acid formation was 29 µg, a result in good agreement with earlier experiments. After the addition of 50 µl of diluted phosphorylase suspension, there was formed 65 µg, and after the addition of 200 µl the lactic acid formation was 73 µg. The lactic acid formation resulting from these two differing amounts of phosphorylase is small and probably not significant. The experiment, however, does clearly demonstrate the effect of phosphorylase. The same homogenate formed under identical conditions 82 µg lactic acid with G-1-P as substrate, and this result is in good agreement with earlier experiments. The addition of phosphorylase partly eliminates the difference in lactic acid formation when G-1-P or glycogen are

Table 34.

Effect of aldolase and of triose phosphate dehydrogenase on lactic acid formation. Standard reaction mixture with fructose-1.6-diphosphate as substrate. 0.3 ml homogenate incubated for 60 minutes. (Exp. no. H-16.)

Aldolase	Dehydrogenase	Net lactic acid formed
ml	ml	
0	0	68
0.05	0	45
0	0.05	265

Table 35.

Effect of the addition of varying amounts of triose phosphate dehydrogenase on lactic acid formation in homogenates of epiphyseal cartilage. Standard reaction mixture. 0.3 ml homogenate. Two different experiments. In exp. H-29, F-1.6-DP and 15 day old rats; in exp. H-30, F-6-P and 13 day old rats.

Dehydrogenase	Net lactic acid formed	
	F-6-P	F-1.6-DP
ml	μg	μg
0	68	173
0.005	210	315
0.010	355	348
0.025	452	353
0.050	442	362

Table 36.

Effect of triose phosphate dehydrogenase on lactic acid formation in homogenates of epiphyseal cartilage with F-1.6-DF and with G-1-P as substrates. Standard reaction mixture. 0.4 ml homogenate incubated for 60 minutes. (Exp. no. H-32.)

Substrate	Dehydrogenase	Net lactic acid formed
	ml	μg
F-1.6-DP	0	246
—»—	0.010	329
G-1-P	0	12
—»—	0.010	9

the substrates. When phosphorylase is added in excess, thus being no longer the rate limiting enzyme, the cartilage is able to form approximately the same amount of lactic acid from the glycogen as from G-1-P, the product of the phosphorylase reaction.

3) *Effect of aldolase and triosephosphate dehydrogenase.* As stated above, the phosphoglucomutase preparation most probably contained other enzymes of the glycolytic cycle. As the lactic acid formation is augmented by F-1.6-DP, this limits the number of enzymes involved to aldolase, triosephosphate dehydrogenase and lactic acid dehydrogenase.

Both aldolase and dehydrogenase were added to the incubating medium containing excess cartilage homogenate. Aldolase did not affect the lactic acid formation, but triosephosphate dehydrogenase augmented it (Table 34). In two experiments where F-1.6-DP and F-6-P were substrates for varying amounts of dehydrogenase, a considerable increase in the lactic acid formation was obtained (Table 35). With G-1-P as substrate dehydrogenase had no effect (Table 36), and this fact provides further confirmation that phosphoglucomutase is rate limiting under these experimental conditions.

The following experiment was performed to confirm that it was a low concentration of triosephosphate dehydrogenase which was in part responsible for the slow rate of lactic acid formation. A standard system with F-1.6-DP as substrate and 0.3 ml of cartilage homogenate from 16 day old rats was incubated at 37° C for 30 minutes. At the end of the reaction, 2 ml 10 % TCA was added and aliquots taken for lactic acid determination. In addition, two 1 ml aliquots were also taken from all the incubation vessels. In one portion the alkali labile triosephosphates were hydrolyzed by adding 3 ml of 3 N NaOH and after a 30 minute interval, the sample was neutralized with 3 ml of 1 N sulphuric acid. The other aliquot was brought to pH 7 by adding a small quantity of strong sodium hydroxide and after a similar period equal quantities of acid and base were added. Both aliquots were analyzed for lactic acid and inorganic phosphate. The results are presented in Table 37. The quantity of triose and hence the equivalent quantity of phosphate was calculated on the assumption that equivalent amounts of the trioses (glyceraldehyde and hydroxyacetone) and lactic acid give the same colour intensity in

the colorimetric reaction (column 3 and 5). The values are consistent and the results are a further indication that triosephosphate dehydrogenase is a rate limiting step in the system employed.

Table 37.

Demonstration of triose phosphate accumulation during incubation of homogenates of epiphyseal cartilage with F-1.6-DP as substrate. For details see text. (Exp. no. H-62.)

Net lactic acid	„Total triose"	„Total triose" — lactic acid	Alkali labile P	Calculated P
µg	µg	µg	µg	µg
91	240	149	52	51
89	245	156	59	54

D. *Discussion.*

The experiments with slices showed that the glycogen present in cartilage is broken down to lactic acid at a slower rate than the glucose which is taken up from the surrounding fluid. This result was confirmed by the homogenate experiments in which less lactic acid is formed from glycogen per unit of time than when hexose phosphates are used as substrates in the glycolytic cycle.

The optimal system used should provide favourable conditions for lactic acid formation (from the G-1-P formed in the phosphorylase reaction) with glycogen as substrate as judged by the results from experiments where G-1-P was used as substrate. In no experiments where glycogen was the substrate was it possible to reach this activity and is was impossible to obtain further improvement of the system by adding the known co-factors adenylic acid and G-1.6-DP, or by changing the composition of the reaction mixture by increasing the concentration of inorganic phosphate or by bringing the pH nearer to the optimum for phosphorylase, or by adding cysteine. The addition of phosphoglucomutase which increased the lactic acid formation when hexose phosphates are substrates, was without effect with glycogen as a substrate. The addition of crystalline phosphorylase resulted

in a lactic acid formation from glycogen which was of the same order of magnitude as with G-1-P as substrate. Under these conditions it seems reasonable to assume that phosphorylase is the rate limiting enzyme in the system used for measuring the rate of glycogen breakdown to lactic acid in homogenates of epiphyseal cartilage.

The maximal value obtained for phosphorylase activity (experiments no. 80 and 81, Table 32) corresponds to a lactic acid formation of 1.0—1.5 mg per gram cartilage per hour, a value which is slightly higher than that found in experiments with slices for the rate of glycogen breakdown. Under the experimental conditions phosphorylase is the factor which limits the rate at which cartilage converts glycogen.

Gutman and Gutman (1941) could demonstrate chemically the presence of phosphorylase in the epiphyseal heads of young rabbits and rats. Homogenates from young rabbits and rats were incubated for different periods in phosphate glycogen mixtures and analysed for inorganic phosphate. The reduction in the concentration of inorganic phosphate was an indication of the phosphorylase activity. The Gutmans obtained the highest activity with epiphyseal pulp i. e. a mixture of cartilage and newly formed bone tissue. With epiphyseal cartilage, dissected out as cleanly as possible from the epiphysis of young rats, they obtained a 17 % reduction in the course of an hour. Under the experimental conditions, this value corresponds to a phosphate reduction of 0.17 mg per g of tissue per hour, and is equivalent to 1.08 mg glucose per g tissue per hour — a value which is consistent with the results obtained in the present work.

The method used, however, to demonstrate phosphorylase activity by measuring the disappearence of inorganic phosphate is rather unspecific and the results have been doubted by Leynse (1952) who was able to reproduce the Gutman result even with boiled extracts.

Glycogen metabolism and phosphorylase activity have been studied in other tissues. Shapiro and Wertheimer (1943) could not demonstrate phosphorylase in the muscles of rats younger than 10 days. Brain tissue from new born rats showed only 60 % of the adult brain phosphorylase activity and phosphoglucomutase was either inactive or absent. In the light of the results with epi-

physeal cartilage, it is possible that fluoride inactivates the mutase, but this problem will be subjected to closer investigation at a later date.

Novikoff et al. (1948) found with homogenates of Flexner-Jobling tumour that hexose monophosphates could be glycolyzed with or without the addition of F-1.6-DP, but they were less effective than the diphosphate by itself. Unfortunately no results were reported with G-1-P as substrate, only F-6-P and G-6-P being tested. Glycogen was without effect in those experiments. The possibility exists that fluoride has inhibited the phosphoglucomutase activity and hence blocked the decomposition of glycogen.

The metabolic properties of epiphyseal cartilage and embryonic and tumour tissue have many features in common, the most characteristic being a high aerobic glycolysis. Warburg (1926) has formulated the relationship as follows: "Kein Wachtstum ohne Glykolyse."

The primary function of epiphyseal cartilage is to serve as a growth apparatus for the longitudinal growth of the bone. The epiphyseal plate retains its ability to grow and its character of embryonic metabolism after the rest of the organism has left the embryonal stage.

Chapter IX.

General Discussion.

A. *The Rate of Conversion of Glycogen in Relation to the Rate of Growth of the Epiphyseal Cartilage.*

It is possible to evaluate the rate of conversion of glycogen in relation to the rate of growth on the basis of the glycogen content in the epiphyseal cartilage and the phosphorylase activity. Previous calculations have shown that each cell passes through the proliferation zone in 20—30 hours. Assuming that the observed maximal phosphorylase activity is constant in each cell during its development and that the synthesis of glycogen only takes place in the proliferation zone, then the cartilage in the course of 20—

30 hours will synthetize at most 30—40 mg glycogen. The mean amount of glycogen in cartilage was 10—12 mg per gram. Histochemically there are many observations in favour of an uneven distribution of glycogen in the epiphyseal cartilage, i. e. there is less in the youngest and oldest cells than in the cells in the transition period between proliferation and hypertrophy. It is reasonable to assume that the glycogen content in this area may be approximately double the mean value i. e. about 20 mg/g and there is sufficient phosphorylase activity to explain such a high content.

The next question is whether the phosphorylase activity is sufficient to explain the disappearance of the glycogen while the cells pass through the hypertrophy zone. (The additional assumption is therefore made that the phosphorylase activity is constant in the cells during development.) The hypertrophy zone is passed through in the course of 7—15 hours, (mean time 11 hours) of which between 3 and 5 hours are spent in the calcifying zone. Glycogen can no longer be demonstrated histochemically in the calcifying zone and this fact suggests that the cells have between 4 and 12 hours (mean 8 hours) at their disposal for the breakdown of the glycogen. With a maximal phosphorylase activity of 1.5 mg/g the cartilage can metabolize 12 mg of glycogen in 8 hours (with a minimal value of 6.0 mg and a maximal value of 18 mg per gram cartilage).

According to these calculations, the phosphorylase activity is more than sufficient to account for the glycogen synthesis, but represents a limiting value for glycogen breakdown. Another possibility is that the phosphorylase activity is not constant in the cells during their development, but that the activity increases with the degree of maturity of the cells. A considerably more refined technique would, however, be required to determine the quantitative distribution of phosphorylase in the tissue. In 1953, Cobb employed a histochemical method to observe phosphorylase qualitatively in slices of epiphyseal cartilage. With this method, which probably requires further modification, it was shown that the phosphorylase activity was highest in the somewhat flattened cells which had just begun to hypertrophy and form columns.

The rate of glycogen conversion is determined by the activity of the phosphorylase system. The direction of the reaction is pri-

marily determined by the concentrations of the substances involved in the reaction:

$$\text{Glycogen} + H_3PO_4 \overset{\text{phosphorylase}}{\rightleftharpoons} \text{glucose-1-phosphate}$$

An increase in the concentration of G-1-P or a reduction in the concentration of inorganic phosphate displace the equilibrium to the left, the converse being also true. The quantity of phosphatase increases with the age of the hypertropic cartilage cells (Follis 1949). Bone phosphatase splits G-1-P and other hexose phosphates at approximately the same rate. Where the phosphatase activity is sufficiently high in relation to the activity of the glycolytic enzymes, the hexose and triosephosphates are split hydrolytically with subsequent decomposition of glycogen. In many respects this type of mechanism recalls that in which the formation of blood sugar from liver glycogen is most satisfactorily explained by the presence of a specific glucose-6-phosphatase (Swanson 1950).

Glycogen accumulates in the younger cartilage cells in which phosphatase activity cannot be demonstrated or is at least very low. Glycogen disappears as the cartilage cells become older and the phosphatase activity increases. There should be a stage in the development of the cartilage cells at which synthesis and breakdown hold one another in check, i. e. a dynamic equilibrium. This equilibrium can be affected by a number of factors. Thus a caloric undernourishment, or almost any other type of nutritional deficiency, displaces the equilibrium in the direction of breakdown. A reduction in cartilage width may be morphologically observed during hunger. The width of the cartilage (thickness of the epiphyseal plate) increases during optimal growth and the effect of the hormone from the hypophysis is well known. Elrick (1953) has shown that purified preparations of the hyperglyceamic factor from pancreas (glucagon) increase the cartilage width of hypophysectomized rats. It is interesting to note in this respect that, in liver, phosphorylase is the rate limiting enzyme for the formation of blood sugar and that glucagon increases the concentration of phosphorylase (Sutherland and Cori 1951).

B. *Glycogen Discussed in Relation to the Calcification of Epiphyseal Cartilage.*

There is a striking correlation in the calcifying zone of the cartilage between the glycogen disappearance and phosphatase appearance. The experiments performed in the present work have not been directly aimed at elucidating this possible causal relationship. In view of the observations made, however, a short discussion is warranted.

When glycogen is metabolized intracellularely via the phosphorlytic cycle, small concentrations of organic phosphate esters are formed which are converted in succession. In the presence of phosphatase these esters can be hydrolyzed. The fact that alkaline phosphatase in bone tissue has a pH-optium nearer the physiological pH, when the substrate concentrations are low, is relevant (Ross et al. 1951). The logical assumption would be that when phosphatase activity is high compared to the activity of the glycolytic enzymes, the phosphate esters which are continuously formed from glycogen are hydrolyzed and no accumulation can occur. Another argument is that any accumulation of phosphate esters would imply that the cell simultaneously had to remove an equivalent amount of phosphate from the surrounding tissue fluids. It would then be unreasonable to suppose that glycogenolysis yields high concentrations of phosphate esters at the site of calcification. A more reasonable assumption would be that the task of the glycogenolysis is to yield phosphate in a form favouring the calcification. This assumption is compatible with the majority of facts found in the experimental investigations.

Neuman et al. (1951) have shown that ester phosphate is adsorbed on bone ash. Before bone crystals are formed, calcium and phosphate are presumably bound in some way or another to the organic intercellular substance of the cartilage. Boyd and Neuman (1951) have shown that cartilage has a binding capacity for calcium, equivalent to its content of chondroitin sulphate. A calcium saturated cartilage in its turn has a binding capacity for phosphate. In addition Neuman et al. have shown that ester phosphate can have an inhibitive effect on *in vitro* calcification when phosphatase is inactivated. They believe that phosphatase splits the phosphate esters which would otherwise inhibit the cal-

cification. There is reason to believe that ester phosphate, during the primary steps of the calcification process, is more readily adsorbed on to the calcium cartilage complex than inorganic phosphate. A better start is thereby given to the calcification than if calcium phosphate was precipitated in an irregular manner. When the nuclei for crystallization are formed, further precipitation of the bone salts can proceed more or less uninhibited by the local factor being mainly determined by the composition of the tissue fluids. The calcification mechanism consists of the following two phases: 1) the primary regulated precipitation or adsorption of the inorganic components and 2) the continued growth of crystals around the crystallization nuclei which are formed.

GENERAL SUMMARY

In the present investigation of glycolysis in epiphyseal cartilage special stress has been laid on the correlation between the activity of the glycolytic processes and the rate of growth.

The experiments were performed with tibiae from rats during the first month after birth. Measurement of the longitudinal growth of the tibia correlated with the histological picture of the proximal epiphyseal plate, permitted calculation of the life span of the cells. Each cell takes 30—45 hours from its development in the cell division zone to its disappearance in the calcifying zone. During development the cells spend 20—30 hours in the proliferation zone, 7—15 hours in the hypertrophic cell zone and 3—5 hours in the calcifying zone.

During the first four weeks after birth, the lactic acid content of tibia remained unchanged (0.6—0.7 mg/g wet weight); the glycogen content fell from 4.25 mg/g after 7 days to approximately 2 mg/g after 28 days. Most of the glycogen was localized in the proximal epiphysis.

Glucose uptake and lactic acid formation in slices of cartilage from proximal tibia epiphysis were measured chemically. At 37° C, 1 gram of cartilage took up 2.5 mg glucose and formed 2.0 mg lactic acid per hour when the incubating fluid contained glucose. The lactic acid formation was over a wide range independent of the glucose concentration in the incubating fluid. Glucose and mannose are glycolyzed at the same rate, while fructose and galactose are glycolyzed at a much slower rate or not at all.

Without the addition of any substrate, 0.5—1.0 mg/g/hour of lactic acid was formed. The lactic acid formation was unaffected by low concentration of fluoride, but was stopped by iodoacetic acid.

The glucose uptake of epiphyseal cartilage slices from rats, which one hour in advance had received 20 units of insulin, increased to approximately 4.7 mg/g/hour; the lactic acid formation acid did not increase.

Lactic acid formation in homogenates of epiphyseal cartilage was studied by means of the LePage system (1948). The effect of different hexose phosphates as substrates was investigated. More lactic acid was formed with fructose-1.6-diphosphate than with the other hexose phosphates. Lactic acid formation with glucose-1-phosphate was inhibited by fluoride and required cysteine for maximal activity.

The addition of purified phosphoglucomutase free from phosphorylase increased the lactic acid formation in cartilage homogenate with glucose-1-phosphate and other hexose phosphates, but not with glycogen as substrate. It was found that the mutase preparation contained other glycolytic enzymes. With glycogen as substrate the lactic acid formation was small and of the same order of magnitude as in the experiments with slices. The addition of crystalline phosphorylase to cartilage homogenate with glycogen as the substrate, increased the lactic acid formation to the same extent as when glucose-1-phosphate was the substrate. It was thus demonstrated that phosphorylase is the rate limiting enzyme for glycogen metabolism in epiphyseal cartilage.

The following relations have been discussed:

1) The avascular epiphyseal cartilage nourishment by diffusion of glucose from the adjacent vascularized tissues in relation to the observed glucose consumption.

2) The observed maximal activity of phosphorylase in relation to the growth rate of cartilage, the synthesis and breakdown of glycogen in the cartilage cells and the life of the cells at different stages in their development.

3) The rate of conversion of glycogen in relation to the calcification processes in the epiphyseal cartilage.

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